Femoral Impaction Grafting

Using Bone Graft Substitutes

Rupen Dattani

Centre for Biomedical Engineering (BME)

Institute of Orthopaedics and Musculoskeletal Science

University College London

Royal National Orthopaedic Hospital

Stanmore, Middlesex

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I hereby declare that this submission is my own work and to the best of my knowledge it contains no material previously published or written by another person except where due acknowledgement has been made in the text.

Rupen Dattani

ABSTRACT

Background

Femoral impaction allografting to reconstitute bone loss during revision hip surgery has shown excellent results. However, limitations with the use of allografts have warranted research to investigate if bone graft substitutes could be a suitable alternative to replace or augment allograft in impaction grafting.

Aims and Methods

The objectives of this thesis were to assess if:

- The use of hydroxyapatite (HA) in various combinations with allograft will be biologically effective and functionally stable using a cemented impaction grafting technique in an ovine hemiarthroplasty model. The different treatment groups were compared by measuring the ground reaction forces and new bone formation.

- The addition of mesenchymal stem cells (MSCs) to allograft, HA or an allograft:HA mixture enhances the amount of new bone formation compared with impaction of the scaffold alone in an ovine metaphyseal femoral bone defect model.

- The architecture of the HA scaffold influences bone formation in an extra-skeletal sheep model.

Results

- HA: allograft mixture of up to 90:10 demonstrated similar functional stability and amount of new bone formation as a 50:50 mixture.

- Addition of MSCs to allograft or a 50:50 allograft:HA mixture enhances the amount of new bone formation compared with unimpacted constructs.

- HA either alone or combined with MSCs induces bone growth only when constructed in block form and not in identical porous granular form.

Conclusion

HA is a suitable bone substitute to augment allograft and may be replace bone graft completely in impaction grafting of a femoral component. This has important clinical implications as HA is readily available, easy to use in surgery and not associated with the adverse effects encountered with allografts. The use of MSCs in the treatment of osteolysis holds great potential but further work is required to assess if this technology is transferable to humans.

CONTENTS

ABBREVIATIONS

In alphabetical order:

AG	Allograft
AL	Average Left lower limb ground reaction force
ALP	Alkaline Phosphatase
AR	Average Right lower limb ground reaction force
BMD	Bone Mineral Density
BMP	Bone Morphogenic Protein
CT	Computed Tomography
DBM	Demineralised Bone Matrix
DDH	Development Dysplasia of the Hip
DMEM	Dulbecco's Modified Eagles Medium
FT	Fibrous Tissue
GFP	Green Fluorescent Protein
GRF	Ground Reaction Force
HA	Hydroxyapatite
IMS	Industrial Methylated Spirits
MAR	Mineral apposition rate
MSC	Mesenchymal Stem Cells
NB	New Bone
NICE	National Institute of Clinical Excellence
NIH	National Institute of Health
OB	Osteoblasts
OBLC	Osteoblastic Like Cells

OC	Osteocytes
OP	Osteogenic Protein
PET	Positron Emission Tomography
PG	Prostaglandins
PMMA	Poly Methyl Meth-Acrylate
PTFE	Polytetrafluoroethylene
SEM	Scanning Electron Microscopy
ST	Soft Tissue
TCP	Tricalcium Phosphate
TE	Tissue Engineering
TGR	Tissue Guided Regeneration
TNF	Tumour Necrosis Factor
THR	Total Hip Replacement
UHMWPE	Ultra High Molecular-Weight Polyethylene

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CHAPTER 1

LITERATURE REVIEW

1.1a Introduction

Total hip replacement (THR) represents the most significant advance in orthopaedic surgery in the twentieth century and ranks as one of the most revolutionary advances in modern medicine (NICE, 2000). It achieves an immediate and exceptional restoration in the quality of life that is comparable only to angioplastic treatment of coronary heart disease (Rasanen et al., 2007). THR is most commonly performed for hip joint failure resulting from the arthropathies. Other indications include intracapsular hip fractures, avascular necrosis, bone tumours, polio, development dysplasia of the hip (DDH) and Paget's disease. Most individuals have an excellent prognosis for long-term improvement in symptoms and physical function.

In the United Kingdom there are nearly 60,000 primary total hip arthroplasties being performed each year (National Joint Registry, 2008). In the USA, there are more than 120,000 THR's performed annually, a 64% increase in the last twenty years (NIH, 1995). The average hospital cost of a primary THR, in the USA, is estimated to be over $11,000 (Iorio et al., 1999). In the UK, THR represents a substantial financial burden to the NHS of around £140 million per year, with each trust spending over £250,000 on the purchase of hip prostheses alone (NICE, 2000). Figures from the National Audit Office indicate that the cost of primary THR procedures in NHS Trusts costs an average of around £3,500 (National Audit Office, 2003).

Over the last two decades, complications associated with THR have declined significantly (Clohisy et al., 2004). Prophylactic antibiotic therapy has reduced infection rates and the use of anticoagulants has lowered the incidence of deep venous thrombosis. However, bone loss following a total joint arthroplasty or periprosthetic osteolysis still remains a significant concern (Harris, 2001). Bone loss following hip

replacement was identified as the major long-term adverse effect with THR, at the NIH Consensus Conference on Total Hip Joint Replacements (NIH, 1995). The incidence of periprosthetic osteolysis or aseptic loosening in many studies is greater than the sum of all the rest of the complications (Harris, 2001). In the Swedish Total Hip Replacement Register, osteolysis accounted for over 75% of the patients undergoing revision hip surgery (Malchau et al., 2002).

1.1b Background

The first description of periprosthetic osteolysis was made by Professor Charnley in 1975, when he observed a "cystic erosion of bone" surrounding a fractured femoral component (Charnley and Halley, 1975). Since then, osteolysis has been extensively reported (Zicat et al., 1995; Clohisy and Harris, 1999; Hellman et al., 1999; Dorr et al., 1997; Rubash et al., 1998). Both the acetabular and femoral components may be affected. The prevalence of aseptic loosening, in most series beyond 10-years, is reported to be between 32% and 62%, depending on the type of prosthesis used (Elting et al., 1995; Clohisy and Harris, 1999; Hellman et al., 1999; Dorr et al., 1997).

1.1c Histology

Initial reports examining periprosthetic tissues around loosened components were noted to contain cement particles (Charnley and Halley, 1975). These tissues displayed a high degree of osteoclastic bone resorption, a high concentration of macrophages, and foreign body giant cells with phagocytosed cement particles, leading to what was called cement disease. Polyethylene particles were not implicated as the cause of osteolysis until cementless components were inserted and subsequently noted to have endosteal osteolysis. Particulate polyethylene has been

recovered in significant quantities from periprosthetic tissues and is now considered to be responsible for osteolysis, forming the majority of debris volume (Campbell et al., 1995; Maloney et al., 1995; Margevicius et al., 1994). Studies of wear debris from periprosthetic tissues characterised by scanning electron microscopy have demonstrated that up to 90 percent of the recovered particles are submicron ultra-high-molecular-eight polyethylene (UHMWPE) particles, with a mean size of about 0.5 micrometer (Jacobs et al., 2001). These particles vary in shape, with spheroid forms being the most common. Fibrillar and globular shapes are also present (Jacobs et al., 2001). Billions of particles per gram of tissue have been found (Jacobs et al., 2001). As light-microscopy resolution is limited by the wavelength of visible light (0.4 to 0.7 micrometer), UHMWPE particles cannot be visualised well with conventional histological techniques. The use of Oil Red 'O' (ORO) staining has been shown to be a sensitive and specific marker in identifying UHMWPE particles on histological sections(Hansen et al., 2002; Schmalzried et al., 1993).

The formation of a 'synovial-like membrane' between implant and bone is fundamental to most theories of aseptic loosening (Goldring et al., 1983). Histological analysis of tissue surrounding loosened components (Fig 1.1) after joint replacement reveals the presence of three distinct zones: (1) a thin synovial layer of lining cells supported by fibro-vascular tissue at the cement surface; (2) a middle layer containing histiocytes (tissue macrophages), giant cells, mononuclear cells (lymphocytes and mast cells) and periprosthetic particles; and (3) a fibrous layer that blends into the marrow spaces between bone. The addition of conditioned media to synovial-like membranes, obtained from patients re-operated due to aseptic loosening, has been shown to produce macrophages and giant cells that stimulate

bone resorption, in vitro, by an osteoclast-mediated mechanism (Ohlin et al., 1990; Athanasou et al., 1992).

Figure 1.1: Histological analysis of tissue surrounding loose components. Hyperplastic synovial membrane adjacent to prosthesis-low power (a); methylmethacralate debris surrounded by giant cells-high power (b) and polyethylene flakes (shiny linear material) surrounded by foreign body giant cell reaction-high power (c) and low power (d) (taken from http://www.gentili.net/thr/osteolys.htm).

1.2 Pathogenesis of bone loss following THR

Normal bone maintenance depends on the balance of bone formation and bone resorption that mainly involves the coordinated function of osteoblasts and osteoclasts. There are several postulated mechanisms by which bone loss after a joint replacement may occur (Rubash et al., 1998).

1.2a Ageing

Bone loss may occur as a result of natural ageing. Females can lose up to one-third of their cortical bone and half of their trabecular bone throughout their lifetime, whilst males lose about half that amount (Rubash et al., 1998). Also, with increasing age, bone resorption occurs throughout the skeleton and about one quarter of total bone mineral density is lost in the proximal femur by the age of seventy (Rubash et al., 1998). However, post-mortem analysis of retrieved femurs in elderly patients treated with cemented femoral implants does not show the ageing process to be a major threat to the mechanical stability of the implants (Coathup et al., 2001; Jasty et al., 1990).

1.2b Adaptive bone remodelling or stress shielding

Adaptive bone remodelling or stress shielding can occur in response to an altered mechanical environment following a hip replacement. It occurs because there is a redistribution of load (and consequently stress on the bone) when the femoral head is replaced by the femoral component of a total hip replacement. Consequently, stress on the proximal femoral cortex is lessened, as most of the load bypasses this area and is transmitted in the metal stem to the distal femur.

Cemented stems are associated with less stress shielding than uncemented stems (Huiskes and Boeklagen, 1989; Harris, 1992). This is probably because uncemented stems are larger and stiffer than cemented stems and both these factors are known to increase stress shielding (Huiskes and Boeklagen, 1989; Rubash et al., 1998). Although, bone resorption in unstressed areas has been a frequent finding, it is not associated with loosening (NIH, 1995). Studies have shown that fully coated stems

are associated with an increased cortical bone stress shielding compared with proximally coated porous stems (Huiskes and Boeklagen, 1989; Weinans et al., 1994). The amount of coating on most prosthetic stems available today is still greater than that necessary to reduce the stress-shielding effect on the proximal femur (NIH, 1995). However, reducing porous coating to lower stress shielding must be balanced against providing adequate coating to ensure fixation. Although the long-term effects of stress shielding on the stability of components and further revision surgery are not known (Rubash et al., 1998), the lack of good bone stock encountered provides a technical challenge for the surgeon.

1.2c Mechanical Factors

Migration of a prosthesis is defined as a change in the position of the prosthesis, cement mantle or both and is thought to indicate implant failure and evidence of loosening (Harris et al., 1982). The mechanism by which migration occurs is not fully understood. Early migration could be attributed to surgical technique (reaming, broaching or high pressure pulsatile lavage), which disturbs the capillary circulation of the periprosthetic bone, leading to necrosis. Other postulated causes include infiltration of loose connective tissue in the process of remodelling and revascularisation of the bone graft (Franzen et al., 1995), poor cementing technique (Iyer and Krishnan, 2006), fracture of the cement-graft complex (Franzen et al., 1995) or distal movement within the cement mantle due to creep of the cement (Fowler et al., 1988). Late migration is most likely due to fatigue-failure of cancellous bone surrounding the prosthesis (Taylor and Tanner, 1997) leading to loss of osteointegration of a stable prosthesis. Once migration has started to occur there is then a race between remodelling of the bone around the prosthesis to restore stability

and progressive loosening. It is not fully understood why some prostheses remain stable whilst others migrate. Once migration has started, stability is lost and periprosthetic particles may modulate the later stages of loosening (Aspenberg and Herbertsson, 1996). Instability has been shown to lead to the formation of tissue similar to fibrous membrane around loosened components (Aspenberg and Herbertsson, 1996). Once this process has started periprosthetic particles allow this membrane to persist and inhibit new bone formation around it (Aspenberg and Herbertsson, 1996).

1.2d Fluid Pressure

Once a synovial-like membrane has been formed, synovial fluid pressure within the joint may cause osteolysis in one of two ways. Firstly, it may contribute to the spread of wear debris which directly initiates bone resorption via macrophage activation (Aspenberg and Van, V, 1998) (Fig 1.2). Secondly, an elevated fluid pressure itself can disturb the normal perfusion and oxygenation of bone resulting in osteocyte destruction and stimulation of macrophages to secrete cytokines that ultimately cause bone resorption (Aspenberg and Van, V, 1998; Ferrier et al., 2000; McEvoy et al., 2002). Synovial fluid pressure can rise up to 700mm Hg during walking, stair climbing or rising from a sitting position but pressures as low as 200mm Hg have been shown to cause osteolysis (Aspenberg and Van, V, 1998). For this reason some authors believe that periprosthetic osteolysis is mainly caused by fluid pressure and to a lesser degree by wear particles (Aspenberg and Van, V, 1998).

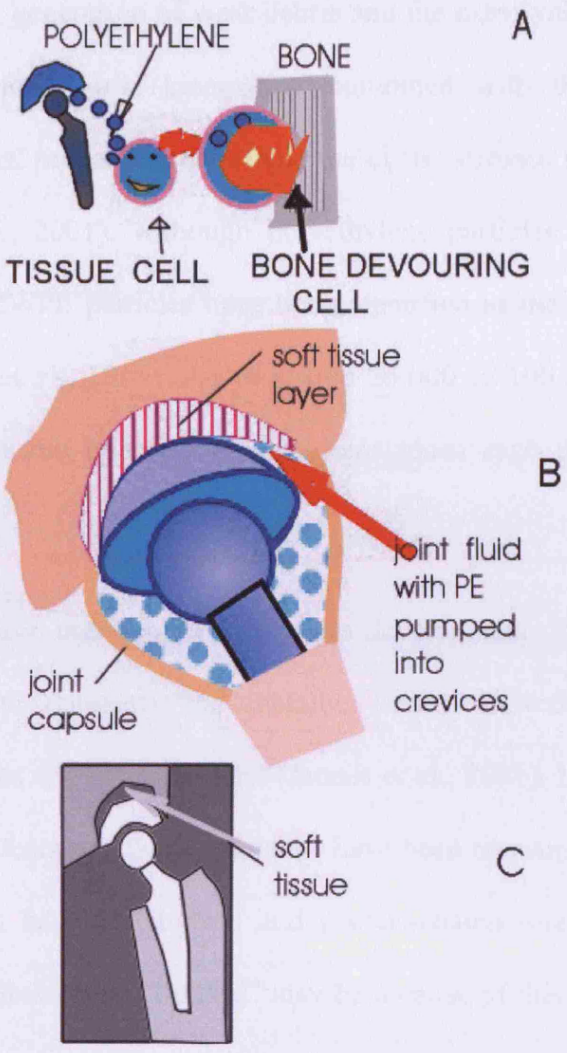


Figure 1.2: Schematic representation showing how synovial fluid pressure within the hip joint causes osteolysis.

1.2e Particulate Debris

It is now widely accepted that bone loss secondary to a biological reaction to particulate debris from implants is the principal mechanism responsible for periprosthetic osteolysis (Rubash et al., 1998). In prosthetic joints, the relevant wear mechanisms include adhesion, abrasion and fatigue. The production of submicron particles in the hip is mainly caused by abrasive wear (Campbell et al., 1995). From a mechanical perspective, implant geometry and material properties are the major

factors influencing the generation of wear debris and the osteolysis that follows (Zhu et al., 2001). Joint forces and kinematics, combined with the contact surface geometries and material properties, determine the cyclic stresses that lead to particle generation (Zhu et al., 2001). Although polyethylene particles are produced in a variety of sizes, UHMWPE particles have been identified as the important cause of osteolysis (Campbell et al., 1995). An estimated 25,000 to 100 million UHMWPE particles are produced and released into the joint space each day (Rubash et al., 1998).

Other particles that have been implicated in the development of osteolysis include polymethylmethacrylate (cement) and metallic debris derived from cobalt and titanium alloys, silicates and stainless steel (Jacobs et al., 2001). High concentrations of metal ions, derived from prosthetic implants, have been measured in the serum and urine of patients who have had hybrid and porous-coated total hip replacements (Jacobs et al., 2001). Stem-bone "fretting" may be a cause of this debris, particularly in distal portions of proximally fixed prostheses where considerable movement between stem and bone may continue, resulting in loosening of prostheses. The metallic particles probably exert their effects by either promoting third body wear of the polyethylene, with UHMWPE triggering the cellular response; or they may directly accelerate the natural cascade which instigates osteolysis. These metallic particles are of particular concern when implants with metal-on-metal bearing surfaces or extensively porous-coated devices with large surface areas of exposed metal are used. Furthermore, metal-on-metal implants have been shown to release high concentrations of metallic ions up to 5 years post surgery (Savarino et al., 2002). The long term biological effects of metallic ions are not known, but concerns have been raised about hypersensitivity and potential toxic effects, including the ability to

cause malignant transformation (Jacobs et al., 1998). However in 1996, Visuri et al. showed no significant increased risk of malignancies 15 years following metal-on-metal articulations.

1.2f Migration of Particles

Particulate matter is dispersed in joint fluid. The concept of an effective joint space, which includes all periprosthetic regions that are accessible to joint fluid and thus particulate debris, has been proposed as the mechanism for migration of particles (Schmalzried et al., 1992). The presence of particulate matter in joint fluid will initiate a localised macrophage-induced phagocytosis and result in bone resorption. As bone is resorbed, a pool is formed, promoting increased flow (preferential flow) into that region and thus delivering more particles and causing further localised bone resorption. This cycle continues and eventually a significant quantity of bone becomes resorbed to become evident as an osteolytic area on a radiograph. Fluid pressure propels joint fluid and thus particulate debris through the effective joint space, resulting in progressive bone loss (Schmalzried et al., 1992).

Small particles (0.5-10μm) promote the most severe inflammatory response because they are phagocytosable and elevated levels will induce an increased immune response (Jacobs et al., 2001). When generated, these small particles will follow a route of least resistance and become interposed between the bone/cement interface or between the bone/implant interface in uncemented prostheses (Jacobs et al., 2001). In cemented femoral components, the path of least resistance is along the cement-metal or cement-bone interface. For example, particles may be driven along the interface between the stem and cement and through defects or cracks in the cement mantle and

provide a route through which the contents of the joint cavity may reach the endosteal surface of the femur, leading to localised bone lysis (Anthony et al., 1990). In addition, particulate debris is transported away from the joint through the lymphatics or urine (Langkamer et al., 1992). The saturation of these systems would result in local particle accumulation and further osteolysis could develop.

1.2g Cellular Response to Particles

The cellular response to particles is complex and not completely understood (Jacobs et al., 2001). The presence of particulate debris initiates phagocytosis by macrophages and macrophage derived foreign-body giant cells. As a consequence, macrophages and possibly other cells, including fibroblasts, release cytokines such as tumour necrosis factor (TNF-α), interleukins (IL-1, IL-6, IL-10), proteolytic enzymes and prostaglandins (PGE2) (Rubash et al., 1998). Osteoblasts may also cause the secretion of specific cytokines by activated macrophages (Rubash et al., 1998). These intracellular mediators induce a complex cellular response, which initiates a focal bone resorptive process mediated primarily by osteoclasts and to a lesser degree by monocytes (Rubash et al., 1998).

Analysis of periprosthetic material and experimental models of debris-induced osteolysis have implicated osteoclasts as being central to the development of osteolysis (Archibeck et al., 2001). The differentiation and function of osteoclasts is tightly coupled to osteoblasts and the method of coupling has been identified as the receptor/activator of nuclear factor-κβ, its ligand, and the osteoprotegerin (RANK/RANKL/OPG) system (Clohisy, 2003). This system has been directly implicated in the development of osteolysis around THRs. In vitro experiments using

cells retrieved from periprosthetic tissue demonstrated induction of RANK and RANKL mRNA by wear particles (Haynes et al., 2001). Activated cells in the interface membrane of failed THRs overproduce both RANKL and RANK (Ohmori et al., 2002). The expression of RANK, RANKL, and OPG in the periprosthetic tissue of both aseptic and septic loosened THRs has implicated this system as being central to the mechanism of bone loss (Gehrke et al., 2003).

1.2h Biological Response to Wear Debris

The presence of wear debris does not always result in osteolysis. For osteolysis to occur, the rate of production of wear particles must exceed an individual's capacity to remove the debris such that a threshold is reached above which the development of osteolysis is more likely (Dumbleton et al., 2002). Furthermore, the normal repair mechanisms that are responsible for preventing the formation of osteolytic lesions are unable to halt the disease progression (Kadoya et al., 1998). Therefore, an individual's biological response to the presence of wear debris may play an important role in the development of osteolysis. This may explain why in some cases osteolysis is not progressive, whilst in others the biological process is more aggressive and eventually results in loosening (Harris, 2001).

1.2i Wear and Osteolysis

Linear wear rate is most often used in studies to measure the presence and development of osteolysis rather than volumetric wear. Linear wear rate is defined as the penetration of the metallic head into the plastic cup liner and is measured on a series of radiographs. A hip bearing wear rate of 0.1mm / year has been suggested as the wear threshold for polyethylene (Dumbleton et al., 2002). The incidence of

osteolysis rises significantly as the linear wear rate rises above 0.1mm/year, whilst lesions are rare at a wear rate of less than this level (Charnley and Halley, 1975).

1.3 Classification of Osteolytic Defects

The defects that result from osteolysis can be classified according to the AAOS system as being cavitary or segmental (Table 1.1, Fig 1.3). Cavitary defects do not compromise the mechanical integrity of the involved skeleton, whereas segmental defects severely compromise mechanical integrity (D'Antonio et al., 1993). Another classification system is based on the location and extent of femoral bone loss (Table 1.2), with the adequacy of the metaphyseal, diaphyseal and cancellous bone in the femur used to predict reconstructive options (Paprosky and Burnett, 2002). For the acetabulum (Table 1.3), a radiographic classification system is based on the antero-posterior (AP) pelvis plain film that predictably classifies the defect found at the time of surgery and allows for preplanning (Paprosky and Burnett, 2002).

1.4 Clinical and Radiographic Manifestations of Osteolysis

Radiolucent lines are seen around the loose prosthesis on radiographs, most commonly in the lateral and anterior aspects of the femur (Masri et al., 1998) (Fig 1.4). Radioisotope scans may reveal areas of increased activity in areas of loosening. Radiographic manifestations of periprosthetic osteolysis around total hip replacements can be classified into two main groups, i.e. expansive and linear (Masri et al., 1998) (Fig 1.5). In linear wear, the radiolucent lines form and expand around the periphery of the prosthesis. Successively, the lines which correspond to the osteolytic process become thicker. The loose tissues, together with the cells that are responsible for the removal of bone, expand successively around the whole

surface of the total hip components. Eventually all surfaces of the prosthesis are in contact with soft connective tissue only and all fixation between the prosthesis and the skeleton is lost, rendering the prosthesis loose. In expansile wear, the osteolytic process starts on a small area and spreads into the skeleton, away from the surface of the prosthesis (Zicat et al., 1995). The skeleton may bulge outward under pressure, thus the name "expansile".

In the majority of cases, radiographic evidence of the disease process only manifests five years or more after the insertion of the prosthesis (Harris, 2001). Clinically, most patients are asymptomatic and diagnosed only following an incidental finding on late post-operative radiographs (Harris, 2001). In a minority of cases, patients are symptomatic and present with thigh pain (usually indicating femoral component loosening), groin pain (usually indicating acetabular loosening) or fractures of the femur or acetabulum.

Category	Description
I	Segmental deficiencies • Proximal Partial (anterior, medial or posterior) Complete • Intercalary • Greater Trochanteric
II	Cavitary deficiencies (Cancellous, Cortical, Ectasia)
III	Combined Segmental and Cavitary
IV	Malalignment (Rotational or Angular)
V	Femoral Stenosis
VI	Femoral Discontinuity

Table 1.1: AAOS Femoral Bone Loss Classification System

Figure 1.3: AAOS Classification system of femoral bone loss: (I) Segmental, (II) Cavitary (III) Combined (taken from D'Antonio et al., 1993).

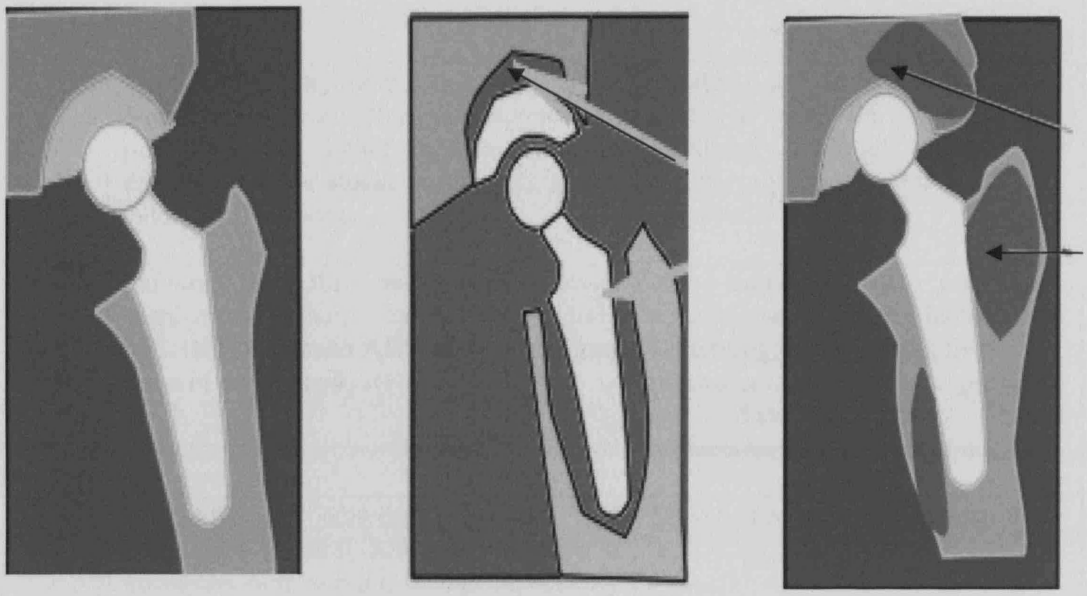


Figure 1.5: Schematic representation of expansile and linear

Figure 1.6: Endo-Klinik classification of femoral bone loss (taken from Gozzard et al. 2003).

Table 1.2: Paprosky Classification System of Femoral Bone Loss (taken from Paprosky & Burnett, 2002).

Table 1.3 Paprosky Classification of acetabular defects (taken from Paprosky & Burnett, 2002)

Figure 1.4: Radiographic appearance of osteolysis. (a) Proximal femur, (b) acetabulum and (c) plain film and 3D segmented CT of periacetabular osteolysis. Regions of osteolysis are indicated by arrows. The virtual reconstruction shows, separately, the femoral and acetabular components of the prosthesis and the pelvic bones. The reconstruction of the region of periprosthetic osteolysis is shown between these two structures in red (taken from arthritis.com).

1.5 Management of osteolysis

1.5a Prevention

The first step in the management of osteolysis is to minimise its occurrence. This can be achieved in several ways. Firstly, design features including modularity, geometry and implant fixation have been shown to be among the most important variables relating to the generation of wear particles (Callaghan, 1995). Therefore, with advances in modern technology and improvements in implant design it is hoped that the prevalence of wear and more importantly osteolysis can be minimised. The femoral head size has been shown to be an important factor affecting wear. With metal on polyethylene articulations, increased head size is associated with increased volumetric wear and a higher cumulative revision rate (Livermore et al., 1990; Eggli et al., 2002). The dimension of the femoral head should be selected to assure a minimum of 6mm and ideally 8mm in polyethylene thickness (Bartel et al., 1986). As for the material, titanium femoral heads should be avoided because they have been shown to wear excessively compared with cobalt chrome, stainless steel or ceramic heads (Agins et al., 1988).

Secondly, it is important to prevent wear debris from gaining access to the bone-implant interface. In cemented femoral components, the path of least resistance for particle migration is along the cement-implant or the cement-bone interface. During normal gait high intra-articular pressures are generated which drives joint fluid and particles towards the cement-bone interface. Any defects or fractures within the cement mantle or debonding at the cement-implant interface will allow the ingression of particulate debris with subsequent osteolysis. The use an efficient cementing technique can diminish the effective joint space and seal the entrance to the femoral

component. This is reflected in the low incidence of osteolysis, at long-term follow-up, with the use of cemented femoral stems inserted using second-and third-generation cementing techniques (Goetz et al., 1994; Mulroy and Harris, 1997). In cementless systems, prostheses that do not have a circumferential porous surface may allow the formation of fibrous channels through which particulate debris may enter (Coathup et al., 2001; Freels et al., 2002). In patch-porous coated implants, the path of least resistance is often along the smooth portion of the stem into the femoral diaphysis (Schmalzried et al., 1992). This is evident in a high incidence of femoral diaphyseal osteolysis noted in many clinical series using patch porous-coated components (Maloney and Woolson, 1996; Hallan et al., 2006). The use of circumferential porous coating aims at creating a seal at the proximal femur since a bioactive surface with osseointegration has an efficient sealing effect (Schmalzried and Harris, 1992). Circumferential porous-coated stems have usually shown low rates of femoral osteolysis at long term follow-up (Bojescul et al., 2003; Xenos et al., 1999; Archibeck et al., 2001).

Thirdly, new bearings for total hip arthroplasty have been introduced with the aim of reducing the number of biologically active wear particles. There are two approaches: one is to improve the wear resistance of polyethylene through cross linking and the other is to avoid polyethylene and utilise alternative bearings.

(i) Cross Linked Polyethylene

Through manufacturing processes, the polyethylene is altered to generate cross-links between the polymer strands, generating a more wear-resistant material. In recent years, there has been a rapid expansion in the use of highly cross-linked polyethylene

in the USA and Europe with some countries using it in nearly two thirds of all their hip replacements (Kurtz SM, 2004). Laboratory wear testing of highly cross-linked polyethylenes in hip joint simulators have shown a significant resistance to wear with some studies reporting over 90% reduction in wear even under adverse conditions, such as with large-diameter femoral heads, with roughened femoral heads, and in the presence of third-body particles (Affatato et al., 2005; McKellop et al., 1999; Muratoglu et al., 2001; Kurtz et al., 1999; Harris, 2004). These findings have been confirmed by retrieval studies of first generation highly cross-linked polyethylene acetabular components (Oonishi and Kadoya, 2000; Oonishi et al., 2001) and randomised controlled trials comparing second generation highly cross-linked polyethylene with standard UHMWPE (Glyn-Jones et al., 2008).

When evaluating the clinical published reports on the use of highly cross-linked polyethylene, two important factors must be taken into account. Firstly, although the wear rate of the highly cross-linked polyethylene during the early years of clinical use have been shown to be lower than that of traditional polyethylenes, the amount of reduction has been lower than that predicted with hip-simulator wear testing (Jacobs et al., 2007). This difference is most likely due to the fact that there is a biphasic pattern of penetration of the ball into the cup; the initial phase is rapid and creep-dominated and the latter phase is slower and dominated by wear (Digas et al., 2004). Therefore, a significant problem with the assessment of polyethylene wear in vivo is the determination of when creep-dominated penetration slows and when wear-dominated penetration begins (Jacobs et al., 2007). Secondly, when the amount of reduction in wear is compared among clinical studies of different highly cross-linked polyethylenes, the type of polyethylene used for the control group must be taken into

account. This is due to the fact that traditional polyethylene cups that are sterilised using gamma irradiation ranging from 2.5 to 4 Mrad (25 to 40 kGy) have a certain amount of cross-linking that, on the basis of hip-simulator studies, would result in wear rates that are about 50-75% lower than those of non-cross-linked polyethylene cups (McKellop et al., 1999).

Some studies have raised concerns about the mechanical properties of highly cross-linked polyethylene which has decreased toughness and elastic modulus when compared with standard UHMWPE (Baker et al., 1999; Baker et al., 2003). Another study has reported wear and surface fatigue cracking, as early as ten months after implantation of a highly cross-linked ultra-high molecular weight polyethylene acetabular liner (Bradford et al., 2004). In addition, hip-simulator results of wear resistance can differ greatly from those of clinical studies (Graeter and Nevins, 1998; Glyn-Jones et al., 2008) and for this reason some authors advocate that clinical trials are essential before highly cross-linked polyethylene becomes so widely used (Glyn-Jones et al., 2008). Furthermore, cross linked polyethylene has been shown to release a relatively large number of wear particles that are more biologically active than that produced by non cross-linked polyethylene (Endo et al., 2001). Therefore, the amount of cross-linking that leads to the least amount of wear debris production is yet to be determined. Although short to mid term studies for cross-linked UHMWPE seem encouraging, longer follow ups are required.

(ii) Ceramic on ceramic

First generation ceramic-on-ceramic bearings were complicated by neck-socket impingement, ceramic fractures, isolated accelerated wear from chipping, and implant

loosening (Campbell et al., 2004). Both impingement and implant loosening were largely design problems and unrelated to the bearing surface (Holmer and Nielsen, 1993). The incidence of ceramic fracture during this period was primarily due to manufacturing flaws, lack of testing standards and poor tolerances for taper designs (Campbell et al., 2004). More recent processing techniques may have eliminated these problems. Newer generation of ceramic components including alumina and zirconia ceramic have a low coefficient of friction and superior wear characteristics compared with metal (Campbell et al., 2004).

Ceramic on ceramic articulations are estimated to have 150-300 times less linear wear and 1700 times less volumetric wear than conventional metal-on-polyethylene articulations (Campbell et al., 2004). Although, in vitro studies have shown that ceramic wear debris is not biologically active (Sterner et al., 2004), there is clinical evidence to demonstrate that it can cause osteolysis (Yoon et al., 1998). Early results with the use of ceramic-on-ceramic hip replacements have shown encouraging results with low osteolysis rates (Murphy et al., 2006; Colwell, Jr. et al., 2007). However, further studies will be needed to show that the wear resistance demonstrated by ceramics in vitro and early clinical studies actually reduces osteolysis in the long term.

(iii) Ceramic on UHMWPE

The penetration rate of ceramic on polyethylene bearing is between 25-50% of that observed in metal on polymer articulations (Wroblewski et al., 1999). Clinical studies have shown impressive long term results with the use of ceramic-on-polyethylene bearing surfaces (Urban et al., 2001). Although wear rates with ceramic on

polyethylene are lower than that reported with metal-on-polyethylene. This reduction is small compared with hard-on hard bearings and metal on highly cross-linked UHMWPE (Campbell et al., 2004). Furthermore, the UHMWPE wear debris and its associated problems are still present which may explain why ceramic-on-UHMWPE bearing has not consistently shown lower rates of osteolysis (Campbell et al., 2004). In clinical practice, the use of ceramic is limited by expense and a small risk (<0.01%) of head fracture (Fritsch and Gleitz, 1996).

(iv) Metal on metal

Metal-on-metal bearings were employed early in the development of THRs and were commonly used until the mid-1970s but were abandoned largely because of high revision rates (Amstutz et al., 1996) and the success of the metal on UHMWPE Charnley prostheses. Early designs were complicated by high frictional torque from inadequate head-cup clearances, which limited lubrication and contributed to subsequent implant loosening (Campbell et al., 2004). Despite this, in 1990, Jacobsson reported the 20-year survival rate of metal-on-metal McKee-Farrar THR to be 77%, which was comparable to the 73% 20-year survival rate of the Charnley hip (Jacobsson et al., 1990). In addition, fewer osteolytic lesions occurred in patients with metal-on-metal THRs than in those with Charnley THRs (Jacobsson et al., 1990).

Recently, metal-on-metal bearings have been reintroduced with improved materials, design, manufacturing and modularity. Metal-on-metal articulations have several attractions that have resulted in a resurgence of their use. These include improved wear resistance and the ability to self-heal, i.e. to polish out isolated surface scratches caused by third body particles. Furthermore, although metal-on-metal bearings produce more wear particles than UHMWPE (Sieber et al., 1999), the metallic wear

debris is smaller (50-500nm) in comparison with those that activate macrophages (0.5-1μm) and so do not evoke an inflammatory response. This is reflected in some clinical reports demonstrating absence of osteolysis with metal-on-metal articulations at a 9 year follow-up (Dorr et al., 2000). However, other authors have reported a femoral osteolysis prevalence of between 2.6-5.5% in patients treated with second generation metal-on-metal hip arthroplasties (Carr and DeSteiger, 2008; Park et al., 2005).

There has been some resistance to using metal articulations because of the evidence that systemic metal levels are higher in patients with these components (Hallab et al., 2000). This is concerning for two main reasons. Firstly, metal hypersensitivity is suspected to be responsible for some cases of osteolysis and subsequent implant failure (Park et al., 2005; Hallab et al., 2000). Secondly, there is concern that persistently elevated metal levels raises the theoretical risk of toxicity and carcinogenicity (Jacobs et al., 1998). Although some authors have showed no significant increased risk of malignancies following metal-on-metal articulations (Visuri et al., 2006), longer term follow-up especially in younger patients is required.

1.5b Treatment of femoral osteolysis

Despite changes in device designs, implants, materials and fixation methods, revision rates remain at around 10% after 10 years, with cemented prostheses having a lower revision rate than uncemented prostheses (NICE, 2000). Most patients with aseptic loosening will, therefore, need to undergo revision surgery. Currently, revision hip replacements account for around 15-18% of total hip arthroplasties in the UK and USA (National Audit Office, 2003; Dixon et al., 2004; Kurtz et al., 2005). Furthermore, at a time when the average life expectancy is continuing to rise, joint

replacements are being performed on ever-younger patients (Dixon et al., 2004). It can therefore be expected that the need for revision hip arthroplasty will continue to rise for the foreseeable future. Indeed, studies have shown that the number of revision THRs will increase by over 137% over the next twenty-five years (Kurtz et al., 2005).

The two main aims of a revision procedure are to achieve immediate fixation and long- term stability. However, the reduction of bone stock available for subsequent implant fixation probably accounts for the inferior results attained in revision surgery compared with the primary procedure (Pellicci et al., 1985; Sporer and Paprosky, 2004; Krishnamurthy et al., 1997). This is partly due to the inadequate amount and poor quality of bone available into which the new prosthetic components can be fixed and partly due to the fact that the existing bone is often not strong enough to support the loads that are placed on the prosthetic components (Amstutz et al., 1982; Salvati et al., 1975; Stauffer, 1982). Furthermore, the bone loss that accompanies aseptic loosening is often extensive and involves many areas in combination (Behairy and Jasty, 1999). In an attempt to overcome this problem, several considerations must be taken into account when managing the patient with osteolysis.

In the asymptomatic patient, factors such as patient age, past medical history, degree and type of bone loss, rate of progression of osteolysis, properties of the implant to be revised, and patient's activity level must be borne in mind. Surgery is usually indicated if bone loss is extensive or progressive (Stauffer, 1982). Curettage and grafting of the defects, stem retention with exchange of the femoral head is a viable option in the asymptomatic patient with a well-fixed stem (Berry, 2003). In the medically unfit patient or in the elderly, a non-surgical approach with regular follow-up is a reasonable.

In the symptomatic patient, surgery is usually warranted and is guided by host factors mentioned above and the extent of femoral bone loss. The main principles in the management of osteolysis are to identify and remove the source of the wear particles, remove the loose components and fill in any defects. In Paprosky Type I defects (Della Valle and Paprosky, 2004) there is minimal damage to the proximal metaphysis and the case can be approached like a primary femoral hip arthroplasty using any primary cemented or uncemented stem (Dunbar et al., 2001). However, even in these cases less than optimal results have been reported with the use of cemented and proximally coated primary hip stems (Sierra and Cabanela, 2002).

In type II defects, there is meta-diaphyseal bone damage with an intact diaphysis whilst in type IIIA defects, the metaphysis is damaged severely; a minimum of 4cm of intact cortical bone is present in the femoral isthmus (Della Valle and Paprosky, 2004). In these cases, the early results of cemented revision of the femoral component were disappointing (Callaghan et al., 1985; Kavanagh and Fitzgerald, Jr., 1987). One reason for the poorer results was attributed to early cementing techniques which failed to obtain adequate mechanical fixation in the sclerotic, smooth intramedullary canals encountered after removal of a failed prosthesis. With the introduction of second- and third-generation cementing techniques and longer stems to obtain cement fixation in the distal undamaged cancellous bone, some authors have shown encouraging results (Mulroy et al., 1995; Smith et al., 1998; Raut et al., 1995; Collis, 1993) whilst, others have shown an unacceptable failure rate and there are no benefits of revision using improved cementing technique (Eisler et al., 2000). The best results have been observed in older patients (Mulroy et al., 1995; Haydon et al., 2004) but in the younger patient the results have been disappointing (Stromberg and

Herberts, 1996; Haydon et al., 2004). Furthermore, in patients who have had revision of failed uncemented femoral components with use of cement, the rate of loosening at mid-term follow-up is even higher than that reported after revision of failed cemented implants with the use of cement (Davis, III et al., 2003).

If a cemented component is used, it should pass the most distal cortical defect by at least two to three cortical diameters in order to minimise strain and creation of stress raisers on the femur (Panjabi et al., 1985). This often involves the use of a long stem prosthesis that extends beyond the isthmus of the femur. However, in this region the effectiveness of the cement-restrictor is reduced and therefore effective cement pressurisation is not achieved (Dunbar et al., 2001). Furthermore, in the presence of a femoral cortical defect, cement can extrude into the soft tissues making it difficult to obtain adequate cement pressurisation. This may further explain why the incidence of loosening and re-revision has been high in many series reporting the results of cemented revision procedures (Eisler et al., 2000; Stromberg et al., 1992). In addition, the bone-cement shear strength is only one-fifth of the shear strength after primary hip arthroplasty (Dohmae et al., 1988). Some authors have advocated that for these reasons, cement should never be used for revision surgery (Wirtz and Niethard, 1997). In modern times, cemented revision of the femoral component has been reserved for the elderly or the patient with reduced mobility but not for the younger patient in whom there is a realistic probability of further revision surgery.

Due to the high rate of mechanical failure observed with cemented revision, many surgeons now use cementless implants (Sporer and Paprosky, 2003). Bone ingrowth into a cementless prosthesis can be achieved using both porous and hydroxyapatite coatings (Bolognesi et al., 2004). Both designs depend on obtaining an initial stability

and subsequent bone ingrowth. The ability to achieve bone ingrowth correlates with the degree of preoperative proximal bone deficiency and the extent to which the femoral canal is filled with prosthesis (Hedley et al., 1988; Harris et al., 1988; Moreland and Bernstein, 1995; Moreland and Moreno, 2001). As the metaphysis is somewhat supportive in type II defects, a proximally-coated, non-cemented implant with diaphyseal stabilisation can be used or distal fixation can be achieved with an extensively porous-coated implant. However, in type IIIA defects, metaphyseal bone is often deficient and sclerotic or avascular and for this reason the results of revision with proximally porous-coated stems have been disappointing (Berry, 2003; Mulliken et al., 1996). An extensively-coated implant is most commonly used for type III defects and is designed to bypass the regions of proximally deficient bone and obtain stability and fixation in more distal femoral bone. Many studies have shown promising mid- and long-term results with the use of uncemented extensively-coated prosthesis for femoral component revision in these defects (Moreland and Bernstein, 1995; Moreland and Moreno, 2001; Paprosky et al., 1999; Weeden and Paprosky, 2002; Ng et al., 2004). One study has shown that reliable femoral fixation can be achieved in up to 95% of patients with type II or type IIIA femoral defects (Sporer and Paprosky, 2003). However, several concerns have been raised about the use of extensively porous-coated cobalt stems. These include: thigh pain, stem fracture, periprosthetic fracture, late component loosening and proximal stress shielding (Moreland and Bernstein, 1995; Moreland and Moreno, 2001). Stress shielding may be reduced with the use of hydroxyapatite-coated stems (Capello et al., 1998; Malhotra et al., 2008).

A Type IIIB defect is characterized by a severely damaged metaphysis with less than 4cm of cortical bone present distal to the isthmus (Sporer and Paprosky, 2003). In a Type IV defect there is extensive meta-diaphyseal damage and the femoral canal is widened; the isthmus is non-supportive and distal fixation is difficult to achieve (Sporer and Paprosky, 2003). In type IIIB defects, a fully porous-coated stem can be used successfully if the femoral canal is less than 19mm (Sporer and Paprosky, 2003). In canals wider than 19mm and in type IV defects, the use of extensively-coated implants has a much higher mechanical failure rate than that seen with lesser defects (Weeden and Paprosky, 2002; Sporer and Paprosky, 2003; Della Valle and Paprosky, 2004). Furthermore, a bowed stem will follow the natural anterior bow of the femur and will not allow independent positioning of the component. As a result, a femur that has a torsional deformity with a femur that has remodelled in retroversion will be at risk for posterior dislocation (Sporer and Paprosky, 2003).

One alternative to attain axial and rotational stability within a deformed femur is to obtain distal fixation with the use of a fluted tapered stem (Hartwig et al., 1996). This technique originally was popularized in Europe with the use of the Wagner implant (Sulzer Medica, Winterthur, Switzerland). The original design was a monoblock fluted titanium tapered stem designed to provide axial stability through a wedge fit caused by the taper while the flutes allow for rotational stability. The results of this type of implant have been acceptable and in some series have shown reconstitution of bone stock (Isacson et al., 2000; Kolstad et al., 1996; Bohm and Bischel, 2001; Bohm and Bischel, 2004). However, known complications include stem subsidence, dislocation and discrepancies with leg length (Bohm and Bischel, 2004). Furthermore, because metaphyseal and diaphyseal bone loss can be highly variable, it

can often be difficult to position the femoral head centre at the correct length, neck offset, and anteversion with the one-piece stem of this type.

The advent of modular femoral components with multiple-sized proximal and distal stems have evolved to achieve maximal fit of the distal tapered against the isthmus and proximal support to preserve any remaining metaphyseal bone. Modular implants essentially offer distal fixation with proximal stability (e.g. Restoration Modular, Stryker, Mahwah, NJ) or proximal fixation with distal stability (e.g. S-ROM, DePuy Orthopaedics, Warsaw, Ind) (Haddad et al., 2007). These options help establish a stable hip centre and correctly tension soft-tissue to prevent dislocation. The intermediate-term results with this type of implant have been encouraging (Bono et al., 2000; Wirtz et al., 2000; Kwong et al., 2003; Christie et al., 2000; Cameron, 2002). However, the long-term results of this type of implant are lacking and it remains to be seen whether modularity ultimately improves survival rates in revision hip surgery.

In the most severely deficient proximal femur, another treatment option includes the use of an allograft-prosthesis composite whereby a long-stem prosthesis is cemented to a proximal femoral allograft but not to the host bone. One study (Blackley et al., 2001) has shown promising clinical and radiographic results at an average of eleven years after revision hip arthroplasty with this technique. However, this technique is technically demanding and expensive. In addition, although the allograft may heal to the remaining host bone, there is concern that with time resorption may occur rendering the composite unstable (Della Valle and Paprosky, 2004). In the elderly or

low-demand patient, a final reconstructive option includes proximal femoral replacement using a, modular bone tumour type of prosthesis.

Despite the many reconstructive options during revision of the femoral component, there are no studies that have directly compared the results of different femoral revision techniques in a prospective randomised fashion. Unfortunately, these types of studies require large numbers of patients and long periods of follow-up making them difficult to accomplish. Furthermore, it is oftentimes difficult to compare the results of reports because the severity of preoperative bone loss seen at the time of revision THR is variable, and many reports do not use a classification system to allow a comparison of the results of different reconstructive techniques when applied to similar clinical situations.

1.5c Acetabular Revision Options

The goals of acetabular revision surgery are to restore the biomechanics of the hip and to restore structural integrity and continuity. The results of cemented acetabular revisions have been disappointing (Amstutz et al., 1982). Cemented reconstruction using allograft has also produced discouraging results (Jasty et al., 1990). Uncemented porous-coated sockets can be used successfully to reconstruct most acetabular defects encountered during revision surgery (Dorr and Wan, 1995). Screws or cages may need to be used to secure the acetabular component into the pelvis. Where major segmental defects are present and prosthetic stability is not possible in host bone, structural allografts are often used.

1.6 Bone grafts and revision surgery

Bone grafts have increasingly been used to replenish the bone loss that occurs with loosening (Gross and Goodman, 2004; Emerson, Jr. et al., 1989; Head et al., 1999). Bone has three unique properties that are essential for successful healing and incorporation of bone grafts (Garbuz et al., 1998). These include osteogenesis (ability of bone to self-generate new bone formation), osteoinduction (ability to recruit mesenchymal stem cells, from the surrounding host, which then differentiate into new bone) and osteoconduction (process of ingrowth of capillaries, perivascular tissue and osteoprogenitor cells from the host bed into the graft structure) where the graft functions as a scaffold for the ingrowth of new bone.

1.6a Autografts

Autografts are considered the gold standard of bone transplantation because they possess all three unique properties described above (Garbuz et al., 1998). They are usually obtained from the iliac crest, femoral head or the fibula of the patient. There are, however, several limitations with the use of autografts: (1) only a restricted amount of bone can be acquired by this technique and this is usually insufficient to fill the large defects that are associated with loosening. (2) Harvesting the graft from the patient's own skeleton can compromise the normal skeletal architecture and the mechanical integrity of the donor site. (3) Donor site complications and morbidity can result in increased patient recovery time, disability and chronic pain (Vaccaro, 2002). (4) The acquisition of bone graft increases operative time and blood loss. (5) Viable cells harvested from the donor site may not survive when they are detached from their vascular supply (Vaccaro, 2002).

1.6b Allografts

Due to the above mentioned problems with autografts, allografts have become an attractive alternative. These are most often obtained from the femoral heads of other patients undergoing THR's, or from the femurs or tibias harvested from fresh cadavers. Although the use of allografts in the form of morsellised chips to fill cavitary defects has had good clinical success (Berry and Muller, 1992; Padgett et al., 1993) their use in revision surgery has shown inconsistent results (Hozack et al., 1996). Unlike autografts, they only possess osteoconductive and limited osteoinductive properties and remodel at a much slower rate (Vaccaro, 2002). This restricted capacity to remodel may be the cause of fractures observed in large weight-bearing allografts and also has substantial practical significance in terms of the method of fixation of allograft to the host bone (Berrey, Jr. et al., 1990).

There are also several other disadvantages of using allografts. For example, up to a fifth of all donated femoral heads have been shown to be contaminated with bacteria (Sommerville et al., 2000). Furthermore, grafts have been known to transmit pathogens to the patient such as hepatitis B, hepatitis C, HIV and may contain prions (Tomford, 1995; Betz, 2002). In addition, bone allografts have been shown to be immunogenic (Bos et al., 1983). In contrast to autografts, allografts in general have a higher incidence of delayed incorporation, non-unions, delayed unions and failure rates (Gross et al., 1995; Bos et al., 1983; Behairy and Jasty, 1999). Allografts are also not viable at the time of implantation and serve only as a passive scaffold for subsequent revascularisation and osseous ingrowth.

Finally, due to the increased use of allografts, demands for cancellous allografts may outstrip supply in the future (Galea et al., 1998). Grafts can be used as particulate

grafts to fill cavitary defects or as bulk structural grafts to support the prosthetic components. Concerns with the use of bulk allografting include that graft union to the host bone can be significantly delayed (Gross et al., 1995) and that they do not actually replenish host bone stock because they remain unviable (Duncan et al., 1998). Structural grafts have to be stabilised to the existing host bone and early revascularisation can compromise the rebuilding process and cause structural inadequacy (Behairy and Jasty, 1999).

1.6c Allograft preparation and storage

Tissue banks use a variety of techniques in bone allograft acquisition and preparation. With the increased risk of disease transmission, gamma irradiation or ethylene oxide are commonly used to sterilise the allografts. However, there are disadvantages with both methods. For instance, gamma radiation can adversely affect the mechanical and biological properties of bone allografts by degrading the collagen in bone matrix (Nguyen et al., 2007). Although ethylene oxide does not affect the mechanical properties of the graft (Davy, 1999), it can impair the bone conductive properties of fresh frozen bone grafts (Aspenberg and Lindqvist, 1998). Furthermore, the residual debris remaining after ethylene oxide treatment can evoke an inflammatory response and for this reason this sterilisation method is not commonly used for musculoskeletal grafts (Stevenson, 1999). Following sterilsation, allografts need to be stored prior to transportation to a medical facility. Commonly, the bone allografts are either frozen or freeze dried. The advantage of freezing the graft is that it reduces the immunologic response without greatly affecting the mechanical properties (Boyce et al., 1999). However, the problem with fresh frozen bone allografts is that they must be continuously stored at low temperatures, including when transported, and they need

to be thawed prior to use. Freeze drying the graft reduces the moisture content to less than 6%, thus allowing storage for up to five years at room temperature (Ibrahim et al., 2004). The disadvantage of freeze drying is that the allograft will need to be re-hydrated prior to use and rehydrating the bone is reported to restore only about 90% of its original properties. This restoration is dependent on physician compliance with the rehydration process (Boyce et al., 1999). The freeze-drying process may also reduce the compressive strength and rigidity of the allograft (Boyce et al., 1999). Furthermore, it has been reported that the incorporation of freeze-dried grafts can be delayed, with revascularisation and callus formation being reduced compared with other forms of allograft (Stevenson et al., 1997).

1.7 Impaction grafting

Another method that has been employed to treat cavitary defects, in the proximal femur, is the use of impaction grafting, whereby morsellised cancellous allograft is impacted into the proximal femur and the revision prosthesis usually cemented into the canal (Fig 1.7). Whilst in structural grafts, bone ingrowth does not usually exceed 2 to 3mm (Enneking and Mindell, 1991; Hooten, Jr. et al., 1996), in impacted morsellised allografts the bone growth distance has been shown to be greater (Ling et al., 1993). Furthermore, this is the only technique, to date, that has been shown to reverse the loss of bone stock caused by osteolysis (Morgan et al., 2004; Morgan et al., 2004).

1.7a History of Femoral Impaction Allografting

Impaction grafting using either autograft or allograft was initially introduced on the acetabular side in the treatment of protrusio acetabuli (Hastings and Parker, 1975; McCollum et al., 1980). It was subsequently used in the treatment of acetabular osteolysis during revision hip (Slooff et al., 1984; Schreurs et al., 1998). The first clinical reports of impaction allografting on the femoral side were in relation to revision with cementless stems (Nelson et al., 1990). Although the cementless method continues to attract some attention (Keblish et al., 1996), the use of morsellised bone with cement has attracted more enthusiasm over the last decade. This was first described by the Exeter group (Gie et al., 1993) and later by another group (Elting et al., 1995).

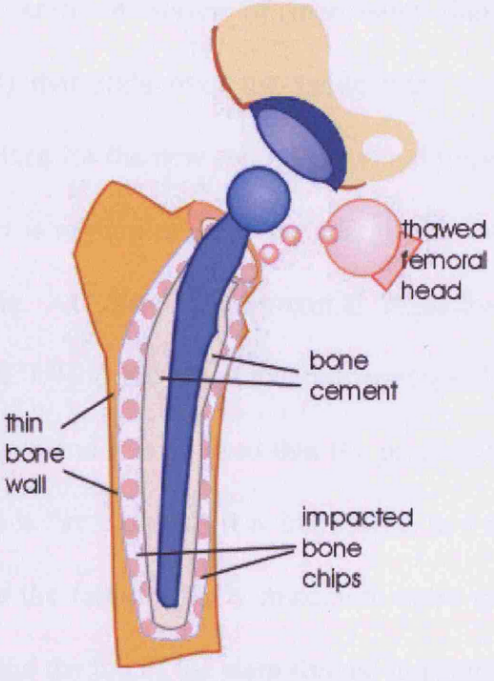


Figure 1.7: Impaction bone grafting of the femur. Allograft is morsellised from donor femoral heads and placed in areas of defect. This is impacted and revision prosthesis cemented in place.

1.7b Technique of Impaction Allografting

The technique of impaction allografting, as described by the originators, has evolved since that reported in the original series from Exeter (Gie et al., 1993). Most techniques now try to maximise the impaction of the allograft, minimise the potential for intraoperative or postoperative fractures, and permit the use of femoral implants with variable stem lengths, neck lengths, and neck offsets (Stulberg, 2002). On the femoral side, the surgery involves removing the old prosthesis, cement and fibrous membrane. Where necessary this is followed by placing cerclage wires or stainless steel mesh around the proximal femur and on lay struts over the proximal cortical defects. The femoral canal is blocked using a special revision polyethylene plug positioned at least 20 mm distal from the anticipated position of the stem tip and a 4 mm diameter guide wire is screwed into the revision plug, which ensures central compaction of the graft. A series of increasing diameter bulbous rods (distal impactors) (Fig 1.8) that slide over the guide wire, are used to impact the graft distally. To create space for the new stem, a proximal impactor (Fig 1.8) is driven into the graft. The former is of similar shape but marginally larger then the stem, to allow for a cement mantle. All distal and proximal impactors attach to a special slap hammer (Figs 1.8 & 1.9) to aid the impaction process. During proximal impaction, graft is added in stages and it is advised that the proximal impactor should be driven into the graft until it is "so tight that it is impossible to withdraw it without using the slap hammer". Once the femur is fully impacted, more chips can be impacted using small hand taps around the top of the stem shaped impactor.

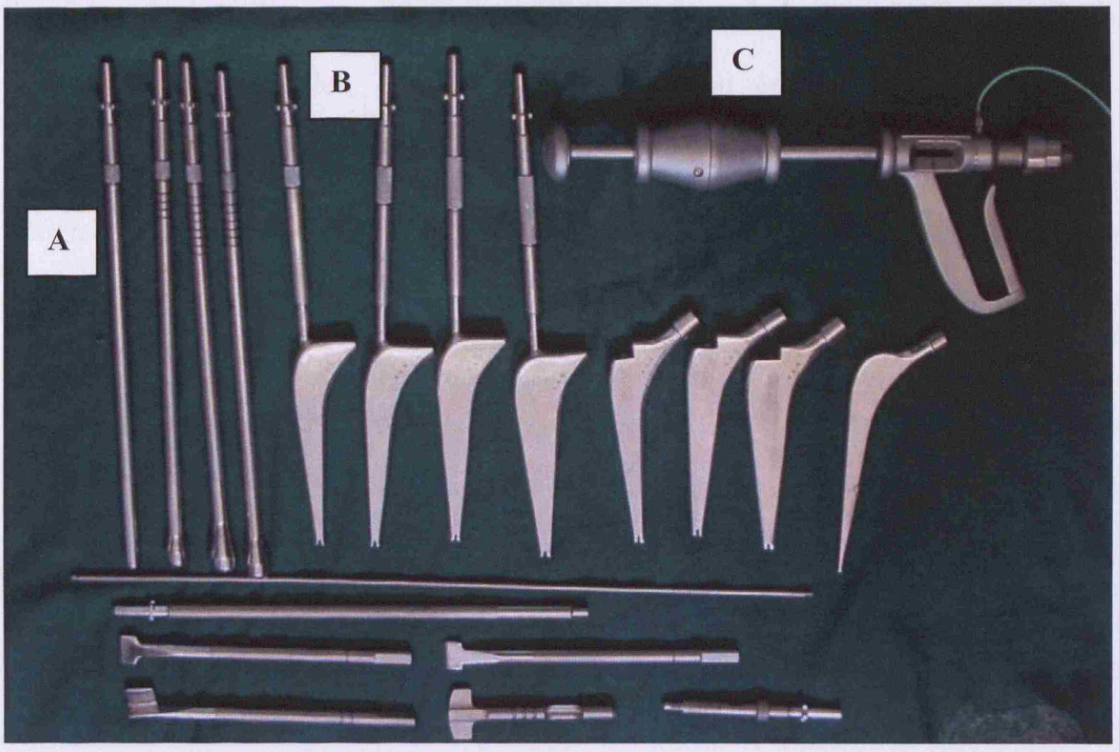


Figure 1.8: Exeter X-change femoral impaction grafting instruments: proximal impactors (A), distal impactors (B) and slap hammer (C).

For revision acetabular reconstruction, the old implant, cement and fibrous interface are removed. Where necessary defects are closed using wire meshes, which are screwed into place (Fig1.10). The bone chips are packed into the small cavities, and then layer by layer the entire socket is filled with the graft using different sized, hand held, domed impactors that can be struck with a hammer (Fig 1.9). The last impactor should be 2 – 4 mm larger than the cup diameter to accommodate the cement used for fixing the cup into position. At least two femoral heads are recommended for acetabular or femoral reconstruction.

1.7c Allograft preparation in impaction allografting

Impaction grafting is most commonly performed using fresh frozen femoral head allograft which is thawed at the time of surgery and milled to the required size. On

the femoral side, the use of a bone mill to produce 2–4mm chips is suggested, however, larger particles, of approximately 10 mm are recommended for the acetabulum (Toms et al., 2004). The most commonly used alternative to frozen allograft is processed bone (freeze dried or irradiated bone). Rarely, xenografts and autografts are utilised but as they are so infrequently used that they will not be discussed further. The surgical technique is the same regardless of the type of graft used.

The use of fresh frozen allograft has been associated with the best long term results (Schreurs et al., 2005). However, the major disadvantage with its use is the possibility of transmission of pathogens to the patient such as hepatitis B, hepatitis C or HIV (Tomford, 1995; Betz, 2002; Simonds, 1993; Conrad et al., 1995). Although processed bone has less potential for disease transmission, there have been concerns that it may have inferior mechanical properties compared with fresh frozen bone (Pelker et al., 1984; Tokgozoglu AM et al., 2000). However, recently in vitro studies have shown freeze-dried graft may provide a more stable fixation of the stem than fresh-frozen morsellised graft (Cornu et al., 2003). Furthermore, the compaction of freeze-dried bone is faster than that of fresh-frozen bone with freeze-dried grafts requiring three to four times fewer impactions to achieve the same stiffness (Cornu et al., 2003). As it is easier to impact freeze-dried bone it may be mechanically more efficient than the fresh-frozen bone in surgical conditions (Cornu et al., 2003). Clinical studies using processed bone have shown inconsistent results (Thien et al., 2001; Buckley et al., 2005; de Roeck and Drabu, 2001; Robinson et al., 2002; Tokgozoglu et al., 2000) and it is not clear if the type of bone graft used or minor variations in technique are responsible for the differences in outcome. However, one

study has shown that the addition of autologous bone marrow to irradiated bone significantly enhances graft incorporation with results comparable to non-irradiated bone (Deakiin & Bannister 2007). Currently, a review to compare the clinical effectiveness of processed bone against fresh frozen bone is being conducted (Board TN et al., 2007).

1.7d Clinical results of impaction allografting for femoral component revision

As with many developments in surgery, the clinical use of impaction allografting has preceded the basic scientific and biological understanding. However, over the last decade, the science behind impaction grafting is slowly being unravelled. When evaluating the published reports on impaction allografting, three important factors limit comparisons among clinical series. Firstly, there is major inconsistency regarding the criteria for inclusion within a study. In some series, only cases with severe advanced bone loss have been included (Masterson et al., 1997; Meding et al., 1997; Leopold et al., 1999), whilst in other studies cases with such severe bone deficiencies have specifically been excluded (Gie et al., 1993; Ling, 1997). In some studies, inclusion criteria have not been defined at all (Eldridge et al., 1997). Secondly, there are a vast number of confounding variables in the published reports that again limit valid comparisons. For example, series have differed with respect to stem geometry, cement type, allograft material, surgical approach and post-operative care of patients (Leopold and Rosenberg, 2000; de Roeck and Drabu, 2001). Finally, methods of patient analysis, including clinical and radiographic assessment and follow-up duration, have differed markedly among published reports (Leopold and Rosenberg, 2000).

Since the inception of this technique in 1985, there has been limited clinical follow-up on cementless femoral impaction allografting, and most follow-up series have evaluated the cemented method. The technique using cement has shown encouraging results and appears to show restoration in bone stock in a majority of patients (Mikhail et al., 1999; Meding et al., 1997; Elting et al., 1995; Leopold et al., 1999; Gie et al., 1993).

In addition, radiological and histological graft incorporation has been reported and the clinical results have been promising regarding pain relief and function (Gie et al., 1993; Emerson, Jr. et al., 1989). However, early subsidence of the femoral component, prosthetic dislocations and a high incidence of intra- and post-operative femoral fractures have been reported (Eldridge et al., 1997; Masterson et al., 1997; Franzen et al., 1995). Several centres have reported short-term (minimum 18-24 months) follow-up using cancellous impaction grafting with cement using polished, tapered, collarless stems (Eldridge et al., 1997; Elting et al., 1995; Gie et al., 1993; Meding et al., 1997). Advocates of the technique using these devices state that subsidence does not automatically lead to clinical loosening because the stem's wedge-shaped geometry may allow restabilisation within the cement mantle as subsidence occurs (Elting et al., 1995; Gie et al., 1993). Cold flow of the cement mantle may help the stem to subside without becoming symptomatically loose (Leopold and Rosenberg, 2000). Subsidence of the wedge shaped stem may also provide a beneficial compressive load to the bone graft (Ling et al., 1993; Nelissen et al., 1995), whilst some other authors have implicated subsidence as a serious complication and the cause of post-operative thigh pain, cement mantle fracture and hip dislocations (Eldridge et al., 1997; Franzen et al., 1995; Masterson et al., 1997; Meding et al., 1997).

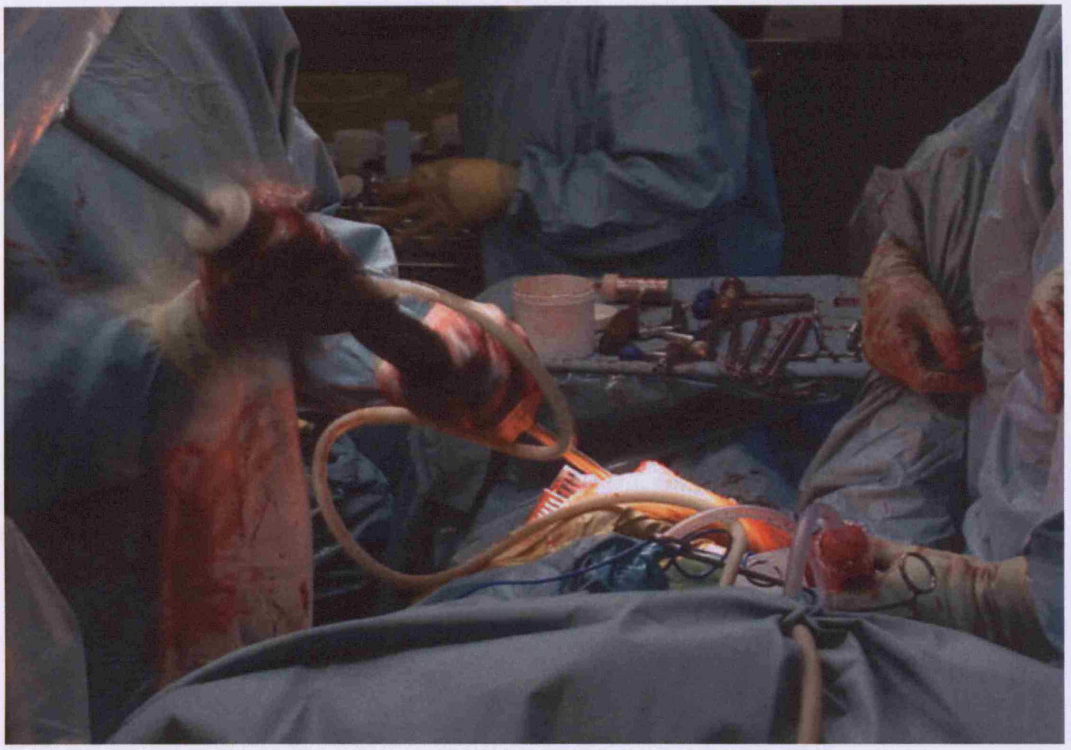


Figure 1.9: Slap hammer used to aid the impaction process.

Figure 1.10: Acetabular reconstruction using impaction allografting. Defects are closed using wire meshes which are screwed into place. (Taken from Gie., et al 1993)

1.7e Evidence for graft incorporation

(i) Radiographic Analysis

The process of bone graft incorporation following impaction allografting can be said to be complete when the graft has been replaced by living bone forming a trabecular pattern with the alignment of the trabeculae associated with the transmission forces from the stem of the femoral component of the distal femur (Duncan et al., 1998). The exact pattern will depend on the design of the femoral component used.

Radiographic inference of preoperative defects and assessment of postoperative graft incorporation poses a major difficulty in the evaluation of this technique. Interpretation of post-operative radiographs is difficult in the presence of overlapping heterotrophic bone, wire mesh, cortical bone, cortical allograft, bone cement and the femoral prosthesis (Duncan et al., 1998; Gie et al., 1993).

There is no universally accepted, reliable and valid radiographic classification system for describing graft incorporation. The originators of the impaction technique proposed a radiographic grading system (Gie et al., 1993), which falls under seven headings: no change; localised resorption; cortical repair; trabecular remodelling; trabecular incorporation; cortical repair and trabecular incorporation. However, there are no other studies, to date, which have validated this grading system. Difficulties in radiographic interpretation of the changes that occur from dead bone graft to fully remodelled, living trabeculae are such that it is difficult to envisage a truly objective means of assessment. However, observed changes are strong evidence for the reconstitutution of living femoral bone stock with this technique (Duncan et al., 1998)

(Fig 1.11). Where cortical and trabecular remodelling are clearly seen, histology from biopsy cores have shown that this corresponds to new bone formation (Linder, 2000).

Figure 1.11: (A) Immediate post-operative radiograph following impaction grafting, (B) magnified image and (C) radiograph at 1 year showing thicker cortex (taken from Linder, 2000).

(ii) Histology

Histological analysis of revised acetabuli with significant bone loss provides most of the indirect evidence for allograft incorporation during femoral impaction allografting. Progressive graft incorporation with subsequent replacement by host bone has been shown in a study on three revision hip arthroplasties in which acetabular defects were filled with impacted allograft bone chips (Heekin et al., 1995). Other human histology studies on the revised acetabulum show revascularisation, new bone formation and reconstitution of the bone stock to varying

degrees (Buma et al., 1996; Heekin et al., 1995; Lamerigts et al., 2000; van der et al., 2002). It is important to note that incorporation of the morsellised graft is often not complete and frequently unpredictable (Tagil et al., 1999). In some instances unincorporated allograft remains, even 8 years after the procedure (Linder, 2000). The incorporation of the graft material depends on the ability of the host osteoblasts and osteoblast progenitors to invade the new scaffold and lay down osteoid, which is subsequently calcified.

Only a limited number of retrieval or revision series have been published to date that evaluate the histological results of femoral impaction allografting (Nelissen et al., 1995; Ullmark and Linder, 1998; Ullmark and Obrant, 2002; Linder, 2000). Of these, only three stem designs have been the subject of histological analysis: the polished tapered CPT stem (Ling et al., 1993), the Lubinus stem (Ullmark et al., 2002), and two versions of the Charnley design (Ullmark et al., 2002; Ullmark and Linder, 1998).

In 1993, Ling et al. published a report on the histology of a single femur retrieved 3.5 years after a cemented revision of a hip replacement in which impaction allografting had been used two fill two large cortical defects. The defects were repaired with a combination of wire mesh and impacted allograft chips, into which a polished, double-tapered CPT stem was cemented. Three distinct histological zones were observed: an inner zone comprising mainly of dead bone trabeculae buried in cement; an interface zone between cement and living tissue; and an outer zone consisting mainly of well vascularised regenerated cortical bone with occasional islands of dead bone. The authors hypothesised that the bone cement forced into the graft immobilises the bone trabeculae at the interface and that the regenerating bone makes

contact with these protruding dead trabeculae. Another study evaluated the histological findings of a retrieved femur 6 months after a cemented cancellous impaction grafting using a Charnley prosthesis (Ullmark and Linder, 1998). This showed that most transplanted areas were revascularised and in the proximal femur there was new bone formation peripherally, but a substantial amount of fibrous stroma embedded graft pieces closer to the cement. In the diaphysis, new bone formation had proceeded to within less than 0.5mm of the cement. Nelissen et al., 1995 reported on histology from four cases that had undergone femoral revision with impaction allografting and in whom the most proximal medial part of the femur had been biopsied during removal of trochanteric wires 11-27 months after revision surgery. Again, three zones were described: an inner zone comprising of necrotic bone and fibrous tissue; an intermediate zone with viable trabeculae (neocortex); and an outer zone with normal cortex and periosteum.

More recently, (Linder, 2000) reported on a histological analysis of 14 cases with duration of 3-96 months after femoral cancellous impaction grafting. The histology varied considerably. Whilst the cortex showed complete reconstitution of viable lamellar cortical bone or ongoing revascularisation of previously necrotic bone, the medullary cavity had a variable combination of trabecular bone, graft particles, fibrous tissue and necrotic graft. This study showed that the initial stage of graft incorporation involved the invasion of the graft, from the periphery, by fibrovascular tissue. This stage did not involve a foreign body reaction. In some sections, avascular impacted graft was observed adjacent to the cement, signifying that the vascular invasion did not always reach the cement surface. Foreign body giant cells were seen on the surface of the tissue in contact with the cement, in cases where the fibrous tissue had reached the cement. New bone formation was only observed in cases where

vascular invasion had taken place. Again three zones were observed; an inner zone consisting of no viable bone but comprised of either composite tissue or necrotic graft; an intermediate zone of intense remodelling with osteoclastic resorption of the surface of graft granules and concomitant deposition of osteoid and bone; and an outer zone with cancellous bone and normal looking bone marrow.

(iii) Increased bone and blood flow formation in impacted morsellised allografts

Sorensen et al., (2003) used positron emission tomography (PET) to evaluate vascularisation and new bone formation in impacted morsellised allograft used in 5 patients revised with femoral impaction allografting. Their results showed much enhanced bone formation and blood flow close to the allograft as early as eight days post surgery. Four months post-surgery, bone formation and blood flow were about the same, but activity was highest in the graft material. Blood flow within the graft bed reduced to levels of the un-operated femoral diaphysis about 1 year after the surgery.

(iv) Animal Studies

In a histological evaluation of impacted allografts surrounding hip prostheses in goats (Schreurs et al., 1994), revascularisation and remodelling of the graft and new bone formation from the cortex towards the cement mantle was observed at six and twelve weeks post surgery. Observations in the titanium chamber model in the rat (Tagil, 2000) resemble those on the histological findings of human femurs (Linder, 2000) in that the fibrovascular tissue penetrates the graft almost completely, whilst new bone formation is more limited. Load bearing has been shown to favour bone formation in a rat tibial prosthesis model (Wang et al., 2000). The addition of OP-1 to impacted

graft promotes bony ingrowth (Tagil et al., 2000) and bone graft incorporation (McGee et al., 2004), in an animal femoral impaction grafting model, proving that biological factors are important in impaction grafting. However, this was a small study using only four sheep, thus making it difficult to draw any real conclusions from it. Furthermore, mixing allograft with OP-1 did not improve cup or stem fixation in revision hip surgery in humans (Karrholm et al., 2006).

(v) Bone-fibrous tissue composite: biological and functional aspects

A recent study in a rat tibial chamber model (Tagil and Aspenberg, 2001) has shown that graft containing fibrous tissue was mechanically stronger than freshly impacted graft. This has lead to conclusions by some researchers (Aspenberg, 2001) that remodelling is not always necessary for a good clinical outcome and that perhaps a combination of necrotic bone and fibrous tissue may constitute an excellent biomaterial for revision surgery.

(vi) Mechanical studies of impacted allograft stability

As well as reconstituting bone stock, any form of grafting should also provide mechanical stability to prevent subsidence and failure of the construct. In vitro studies, using a fibreglass femoral model, have shown that the optimum chip size that produced the greatest stability was around 4-5mm. Other studies have shown that graft with a broad distribution of particle size exhibited superior mechanical properties compared with a graft of uniform size distribution (Tanabe et al., 1999; Brewster et al., 1999). It is noteworthy that bone mills in current use in the UK, produce a distribution of particle sizes more uniform than is desirable for maximising resistance to shear stresses (Brewster et al., 1999).

Mechanical properties of impacted allografts have also been shown to improve with increasing normal load, increasing shear strains, increasing compaction energy and with the addition of small and very small fragments (Brewster et al., 1999). Visco-elastic materials exhibit stress-strain behaviour that is time- and rate- dependent, i.e. the material deformation depends on the load and its rate and duration of application. Creep is the term used to describe an increase in deformation or strain that occurs when a constant load is applied over an extended period (viscoelastic creep). The impacted graft has also been shown to exhibit visco-elastic properties (Giesen et al., 1999) and laboratory studies have shown that this visco-elastic behaviour was affected by several factors. Firstly, reducing the water content and, more significantly, the fat content reduced creep and deformation of the graft (Voor et al., 2000). Secondly, extraction of bone marrow from the bone graft increased stem stability by significantly reducing proximal-distal migration of the stem under loading (Hostner et al., 2001). Finally, a lower graft porosity and stiffer bone graft decreases the visco-elastic behaviour of bone grafts (Verdonschot et al., 2001). Biomechanical studies have shown significantly less stem subsidence in the impacted cortical morsellised graft compared with impacted cancellous graft (Kligman et al., 2002; Kligman et al., 2003).

1.7f Technique Vs System

Some reports of the impaction grafting technique (Eldridge et al., 1997; Elting et al., 1995; Meding et al., 1997; Nelissen et al., 1995) imply it is a system requiring not just an exact surgical method, but also a particular implant, the polished double-tapered stem. Others consider it a surgical technique and have varied the femoral component design (Hostner et al., 2001; Leopold et al., 1999), the method of graft

delivery and any other elements of the procedure (Masterson et al., 1997). In the absence of randomised, controlled clinical trials to evaluate particular elements of this procedure, it is difficult to determine which are the crucial factors. However, investigators have shown similarly good short-term to medium-term results with various femoral stems at numerous centres (Morgan et al., 2004).

1.7g Graft incorporation

The process of cancellous graft incorporation (or healing) is similar to that of fracture healing (Tagil et al., 2000) and is divided into three indistinct stages. Initially, a haematoma forms at the graft site, which is followed by an acute inflammatory reaction (Stage I). The clot attracts platelets, which release inflammatory mediators, which in turn recruit leucocytes and macrophages. In stage II, consolidation occurs whereby fibrovascular tissue invades the graft with subsequent recruitment of mesenchymal stem cells. These pluripotent stem cells differentiate into osteoblasts and osteocytes. Osteoclasts begin to resorb the graft whilst the osteoblasts begin to lay down the osteoid. Remodelling (Stage III) is mineralisation and further maturing of bone.

Cortical graft incorporation differs from that of cancellous grafts. Due to the dense structure of cortical bone compared with cancellous bone, cortical grafts remodel slowly and seldom completely. This process is initiated by an osteoclast-mediated resorption (creating cutting cones through bone) followed by fibrovascular stromal invasion and an osteoblast mediated laying down of new bone. Some reports indicate only 2-3mm of ingrowth in massive cortical allografts (Enneking and Mindell, 1991). Conversely, cancellous bone graft can be completely resorbed. The three dimensional

and porous nature of cancellous grafts is conducive to osteoconduction, which eventually lays osteoid on trabeculae.

1.8 Bone graft substitutes

Due to the previously mentioned complications with the use of autografts and allografts, there has been increasing interest in the use of bone graft substitutes to reconstitute bone loss encountered during revision hip surgery.

1.8a Demineralised Bone Matrix

The use of demineralised bone matrix (DBM) is popular in the USA but it is used to a lesser extent in Europe. DBM is formed from autologus bone extracts consisting of non-collagenase proteins, bone growth factors and collagen. DBM is osteoconductive, moderately osteoinductive but offers no structural support (Gamradt and Lieberman, 2003). DBM becomes biologically inactive at room temperature after a few hours (Delloye et al., 2003). Ethylene oxide and radiation treatment reduces the osteoconductive potential (Delloye et al., 2003). Furthermore, often potentially nephrotoxic chemicals have to be added to the demineralised bone to enable handling and increase volume (Delloye et al., 2003).

1.8b Xenografts

Although xenografts are commercially available, they are rarely used today due to the possible risk of transmitting zoonotic diseases and the lack of evidence supporting their ability to form living bone (Delloye et al., 2003).

1.8c Hydroxyapatite

A variety of bone-graft substitutes, including titanium fibremetals, collagen, bioactive glasses and ceramics composed of hydroxyapatite (HA), tricalcium phosphate (TCP) or both, have also been used to overcome the problems that have arisen with bone grafts (Bucholz et al., 1987). HA and calcium phosphates have been shown to evoke a biological response similar to bone and show great potential as bone-graft substitutes, particularly in contained bone defects (Delloye et al., 2003). Furthermore, they can be readily moulded into desired sizes and shapes and the porous nature of these materials facilitates bony ingrowth. In addition, these materials are osteoconductive, biocompatible, easily sterilised, are not immunogenic and can be used when large amounts of bone graft are not available. However, disadvantages include a lack of osteogenic and osteoinductive potential and their limited ability to offer immediate structural support (Delloye et al., 2003; Bruder et al., 1998).

In non-human primates, hydroxyapatite is the only bone substitute agent which has demonstrated significant osteoinduction heterotrophically on its own, without the addition of bone morphogenic proteins (BMPs) (Ripamonti, 1991; Ripamonti, 1992; Ripamonti et al., 1993; van Eeden and Ripamonti, 1994). It has been postulated that porous HA acts as a substratum for adsorption of endogenously produced BMPs, which then induce bone formation (Ripamonti, 1991; Ripamonti, 1992; Ripamonti et al., 1993; van Eeden and Ripamonti, 1994).

Oonishi et al. (1997) have reported their results on the use of granular HA to fill massive acetabular defects during revision total hip arthroplasty. These show that although on radiographs some spaces were observed at the interface between the HA granules and bone immediately post-surgery, they gradually disappeared within 3

months, presumably because new bone entered the space between the granules encircling the cavity and became bound to them. Furthermore, sclerotic bone that had surrounded the loose cup was seen to change to the appearance of cancellous bone over a period of one to three years after revision.

1.8d Hydroxyapatite and impaction grafting

In vitro studies (Verdonschot et al., 2001) evaluating the mechanical properties of HA/TCP particles in relation to morsellised bone graft for use in impaction grafting have shown that the biomaterial particles do not crunch or damage after impaction. Furthermore, the elastic and viscoelastic deformation of the biomaterials is minimal (Verdonschot et al., 2001). The initial stability of acetabular cups has also been shown to be augmented with the addition of TCP/HA particles in bone impaction grafting (Bolder et al., 2003).

1.9 Summary & Thesis Background

The need for revision hip arthroplasty will continue to rise for the foreseeable future. The principal aims of revision hip surgery are to achieve immediate fixation and long- term stability and to reconstitute bone loss. However, reduction of bone stock available for subsequent implant fixation probably accounts for inferior results attained in revision surgery compared with the primary procedure. The technique of femoral impaction allografting to reconstitute bone loss during revision hip surgery has shown excellent results. However, limitations with the use of bone grafts has warranted this research to investigate whether bone graft substitutes can be a comparable and suitable alternative to replace or augment allograft in impaction grafting.

Hypothesis

This project will test the hypotheses that:

- The addition of hydroxyapatite (HA) in various combinations with allograft induces similar patterns of bone formation as allograft alone.
- The addition of mesenchymal stem cells (MSCs) to allograft or HA enhances the amount of new bone formation compared with impaction of the scaffold alone.
- The architecture of the HA scaffold influences the proliferation and differentiation of MSCs.

Although this study will concentrate on femoral impaction grafting, the results should be relevant in the use of this technique for the acetabulum, as the two are interlinked.

CHAPTER 2

IMPACTION GRAFTING OF FEMORAL STEMS USING HYDROXYAPATITE (APAPORE)

2.1 Introduction

Impaction grafting is a successful technique in reconstituting bone loss during revision hip arthroplasty. However, the numerous complications associated with the use of bone grafts, combined with the fact that demand for cancellous allografts may outstrip the supply in the future, has prompted research to investigate the use of bone graft substitutes (Silva et al., 2005; Pratt et al., 2002). One study (Silva et al., 2005) has highlighted the potential of porous hydroxyapatite to heal bony defects over cancellous bone graft at 24 weeks. The chemical composition and crystallographic structure of hydroxyapatite, similar to bone, makes it a stable, non-toxic material, whilst its macroporosity allows migration of blood vessels and the ingrowth of bone tissue. It is believed that the poor structural integrity of hydroxyapatite and inferior mechanical properties, in comparison to bone graft, limit its use in reconstructive hip surgery (Verdonschot et al., 2001). However, amalgamation with allograft bone in impaction grafting may present a number of potential advantages (Verdonschot et al., 2001; Silva et al., 2005).

ApaPore 60 (Apatech Ltd) is a porous hydroxyapatite that has been chemically and structurally modified to optimise host bone ingrowth in impaction whilst maintaining structural integrity. It has a pore size of 230 μm and porosity of 60%. These granules have interconnected porosity with a macrostructure similar to cancellous bone. The large macropores facilitate angiogenesis and maintain long-term osseous integration (Eggli et al., 1988). Furthermore, deformation of the pores does not result in closure of the pores due to its large size. Its porous structure is consistent, providing a controlled macroporosity within +/- 1%, giving a much more predictable bony response. The struts are highly microporous and the microporosity is interconnected, which allows nutrient transfer to host bone and stimulates cell differentiation (Eggli et

al., 1988). ApaPore has high levels of interconnectivity between micro and macropores. This network allows rapid bone ingrowth, promotes revascularisation, which is essential to healthy bone, enhances long-term graft stability and provides a continuous host bone/graft composite.

Actifuse (ApaTech Ltd) is a phase-pure 80% porous calcium phosphate material in which the phosphate groups have been selectively replaced with silicate ions. Studies have shown that silicon is a significant dietary trace element for healthy bone formation and remodelling (Carlisle, 1981; Schwarz and Milne, 1972). Silicon, released in vivo, is reported to enhance osteoblastic differentiation and stimulate the formation of bone tissue (Patel et al., 2002). Moreover the surface chemistry of Actifuse leads to enhanced adsorption and activation of the proteins involved in osteogenesis, leading to much more rapid and better quality bone formation than with hydroxyapatite alone (Hing et al., 2006).

Besides an improvement of biomechanical properties, expansion or replacement of allograft with synthetic agents would have biological and economical advantages. Indeed, one of the current limitations of impaction grafting is that it relies on the use of allogenic bone. Substituting bone with synthetic granules would address the concerns over transmission of pathogens and shortage of supply (Galea et al., 1998).

2.1a Hypothesis

The use of ApaPore in various combinations with allograft and Actifuse will be biologically effective and functionally stable using a cemented impaction grafting technique in an ovine hemiarthroplasty model.

2.1b Objectives

Hydroxyapatite has proven biocompatibility and the ability to act as an osteoconductive material and when combined with donor bone in a weight relation of 50:50 reduces the required amount of donor bone by half (Verdonschot et al., 2001; Moore et al., 1987; Buckland T. and Lawes P., 2002). However, similar results may be achievable with even higher concentrations of hydroxyapatite, thus reducing the amount of required allogenic bone. Additionally, silicate-substituted hydroxyapatite has been demonstrated to enhance osteoblastic differentiation and stimulate new bone formation compared with phase pure hydroxyapatite (Patel et al., 2002; Hing et al., 2006). The objective of this study was to compare a 50:50 HA(ApaPore):allograft mixture with higher concentrations by volume porous HA (ApaPore) with allograft and Actifuse for cemented impaction allografting of the femoral stem. The four treatment groups used were:

Group 1: ApaPore: allograft mixed 50:50.

Group 2: ApaPore: allograft mixed 70:30.

Group 3: ApaPore: allograft mixed 90:10.

Group 4: ApaPore: Actifuse: allograft mixed 80:10:10.

The four treatment groups will be compared by examining:

a) The **Ground Reaction Force (GRF)** passing through both the operated and non-operated hind limbs pre-operatively and at 8, 16 and 24 weeks post surgery. GRF results are a measure of implant function and how well the animal uses its operated limb and a persistent

reduction in function can be evidence of implant loosening. Furthermore, a failure to regain function after surgery implies that the loads passing through the implant and the associated micromotion may not be representative of a normal healing process which gives anomalous results in terms of bone ingrowth.

b) **Mineral apposition rates** (MAR) within each of the experimental groups. This will allow quantification of the rate of new bone formation within each of the groups, thus permitting comparison of the osteogenic potential of the different bone graft substitutes.

c) The **interface between graft material and tissue** in the different experimental groups in the proximal, mid and tip region of the femoral stem and distal to the tip. As different areas of bone exhibit variable remodelling rates, new bone formation and percentage of remaining hydroxyapatite were measured in these sections.

2.2 Methods

2.2a. Preparation of cancellous allograft and hydroxyapatite

Long bones were removed from healthy sheep that were euthanased as part of an unrelated study. The bones were stored in double-layer polyethylene wrappings at a temperature of –20°C. All soft tissue and cartilage was removed and cancellous portions of long bones were cut into smaller pieces using a bandsaw and then morsellised using a bone mill[1] (Fig 2.1). This created morsellised chips between 3-5mm in diameter. A protocol devised by the Tissue Service of National Blood Service[2] was followed for washing and defatting the graft (Table 2.1, Fig 2.1). The morsellised chips were stored in double-layered airtight polyethylene bags at a temperature of –20°C (Fig 2.1). HA granules (ApaPore 60™ and Actifuse™) were obtained from ApaTech Ltd.

Step	Procedure
1	1 x 15 minutes sonic water wash (hot tap water ~ 55°C)
2 – 4	3 x 15 minutes stirred water wash (hot tap water ~ 55°C)
5	1 x 60 minutes stirred water wash (hot tap water ~ 55°C)
6	1 x 10 minutes stirred and sonic 70% alcohol wash
7 – 8	3 x 15 minutes stirred water wash (hot tap water ~ 55°C)

Table 2.1: Protocol for washing and defatting allograft (devised by the Tissue Service of National Blood Service)

[1] Lere Bone Mill, DePuy International, Leeds, UK
[2] North London Tissue Bank, Deansbrook Road, Edgware, Middlesex, HA8 9BD

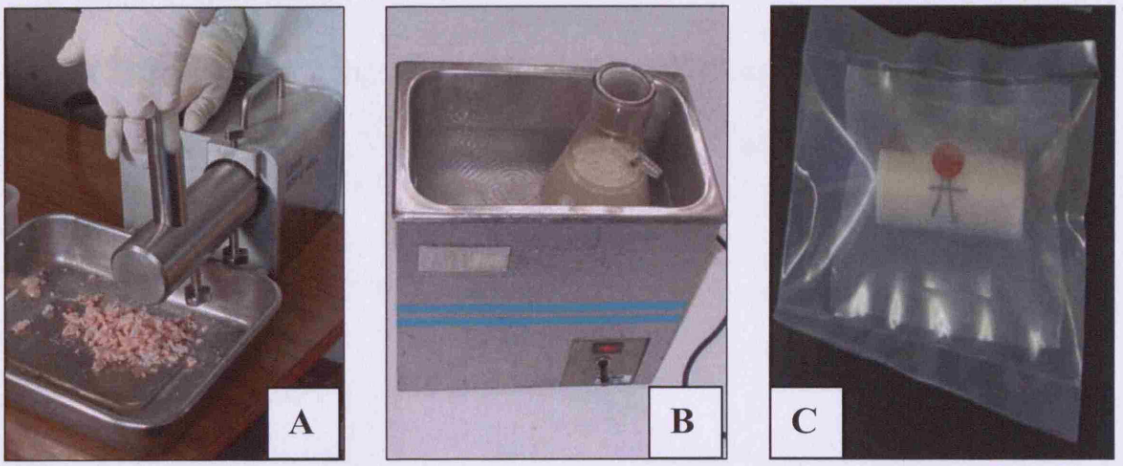


Figure 2.1: Allograft preparation: morcellising in Lere Bone Mill (A), washing in sonic water wash (B) and storage in double-layered airtight polyethylene bags (C).

2.2b Graft Irradiation

Allograft and HA samples were sterilised by gamma irradiation at a dose of 30–35 KGrays[1]. Allograft was stored in dry ice when transported for sterilisation and the irradiated allograft was returned to our laboratory within 48 hours, where it was stored at -20°C.

2.2c Graft preparation

Using stocks of allograft and HA, four treatment groups were produced for implantation (see below). Randomisation of allocation allowed comparison between treatment groups. Six animals in each group were investigated.

Group 1: ApaPore: allograft mixed 50:50.

Group 2: ApaPore: allograft mixed 70:30.

Group 3: ApaPore: allograft mixed 90:10.

Group 4: ApaPore: Actifuse: allograft mixed 80:10:10.

[1] Isotron, Reading, UK

2.2d Femoral stem design

Femoral impaction grafting was performed with a custom designed[1] collarless, polished, double-tapered, Vitallium[2] cemented stem and antibiotic impregnated cement.

2.2e Surgical procedure

All procedures were carried out in compliance with Home Office regulations (Animal Scientific Procedures Act (1986)). Twenty-four skeletally mature female commercially cross-bred sheep weighing between 65 and 80kg and aged between 2 and 5 years were used. The animals were individually housed in pens, forty-eight hours prior to the surgery. Only fluid intake was permitted twelve hours before the operations. Premedication constituted of an intramuscular injection of 2% Xylazine Hydrochloride solution (Bayer PLC, Germany) at a dose of 0.2mg/kg, administered 30 minutes before anaesthesia. Induction of anaesthesia was achieved with an intravenous bolus of 2mg/kg Ketamine and 2.5mg Midazolam mixed with Xylazine (Roche Products, UK) into the left jugular vein and maintained by inhalation of Halothane (Meriel Animal Health Ltd) and oxygen via an endotracheal tube. Buprenorphine (0.6mg) (Reckitt and Coleman Products Ltd) was given intramuscularly as an analgesic at 10-20 μg/kg. Antibiotics (10mg/kg ceftiofur, Pharmacia, Northampton, UK) were administered subcutaneously at anaesthetic induction and repeated once daily for three days. Vital signs (heart rate, blood pressure, respiratory rate and oxygen saturation) were monitored throughout the

[1] Stanmore Implants Worldwide, Centre for Biomedical Engineering (UCL), Royal National Orthopaedic Hospital Trust, Brockley Hill, Stanmore, Middlesex. UK
[2] Cobalt Chromium alloy

operative procedure. A gastric tube was inserted at induction to prevent abdominal bloating, regurgitation and reflux of the rumen contents.

In the anaesthetic room, a large area over the antero-lateral aspect of the right hip was shaved and thoroughly cleaned with Povidine solution. Before commencing surgery, the operative area was further cleaned with a sterile swab soaked in chlorhexidine solution and wiped along the line of the proposed incision, gradually extending across the cleaned area centrifugally in ever-widening circles so that the incision line was not contaminated with a swab that has touched skin at the periphery. Sterile skin towels were applied to isolate the incision line and held together with towel clips.

The animals were placed in a left lateral recumbancy position on the operating table in order to gain exposure to the right hip. The hip joint was exposed through an antero-lateral approach and the gluteal muscles detached partially from the femur. A T-shaped capsulotomy was performed and the femoral head dislocated, then resected. Cancellous bone within the proximal femur was removed using a curette until the largest impactor was able to pass into the femur. A centralising guide wire was attached to the restrictor and the distal graft was impacted in place using modified Exeter impactors that fitted over the guide wire. The femoral canal was blocked using a bone plug positioned at least 20 mm distal from the anticipated position of the stem tip and a guide wire screwed into the bone plug, which ensured central compaction of the graft. Prior to insertion, the relevant volumes of ApaPore granules and allograft were mixed in blood taken from the jugular vein and allowed to clot. Once coagulated, the mixture was inserted and impacted within the femur (Fig 2.2). The graft was then impacted into the walls of the femur using a series of phantom stems which were inserted over the guide wire, ensuring correct rotational alignment. After

the final phantom and guide were removed, the cavity was filled with surgical grade Simplex bone cement. The Exeter styled implant, fitted with a plastic cap to prevent tip loading, was inserted into the cement. In all cases a 25mm diameter femoral head was used. The hip joint was reduced and the soft tissues closed in layers with an absorbable suture.

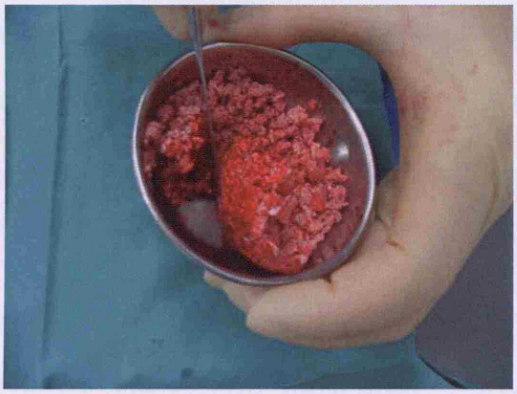


Figure 2.2: A mixture of ApaPore: allograft set in coagulated blood prior to insertion into the femoral canal.

Postoperatively, animals were allowed to fully weight-bear as tolerated. Antibiotic and analgesic prophylaxis was administered daily with subcutaneous injections of Baytril (Enrofloxacin 5mg/kg; Bayer AG Leverkusen) and Finadyne (Flunixin Meglumine 2mg/45kd; Schering-Plough Ltd) and continued for 3 days post-surgery. Animals were kept in individual pens for 4 weeks following the procedure before being group housed. All sheep were euthanased 6 months after surgery with an overdose of intravenous Pentobarbitone (50ml of 20% solution). The femur was harvested through the old incision.

2.2f. Fluorescent Bone Marking

Fluorescent bone markers allowed quantification of new bone formation. They were administered on day 28 and at 2, 3 and 5 months post-surgery. Mineral apposition rates in bone adjacent to the femoral component were compared in the different experimental groups. Prior to staining for histological analysis, thin sections were observed using fluorescence microscopy and pictures taken of the fluorochrome labelled lines. Mineral apposition rates ($mmday^{-1}$) were calculated by measuring the distance between the cement lines and dividing by the magnification of the microscope and the number of days between doses.

2.2g. Ground Reaction Force (GRF)

Animals were walked over a piezoelectric force plate (Kistler) preoperatively and at 8, 16 and 24 weeks post surgery. Twelve readings of maximum force (F_{max}, N/m^2) were taken for the right and left lower limbs of each animal. Average values of right (AR) over left (AL) limbs were calculated as a percentage (%AR/AL) and represent how well the animal used its operated limb, whereby 100% represents full weight-bearing. Preoperatively, only animals with an AR/AL within the 95-105% range were included in the study and all values were normalised.

2.2h. Radiographic Analysis

Each hip joint was radiographed immediately postoperatively, at 8 and 16 weeks post surgery and following sacrifice.

2.2i Histological analysis

Immediately after retrieval, samples were placed in 10% formaldehyde[3], then dehydrated using ascending concentrations of methylated spirit[3] and defatted using chloroform[3] and finally embedded and cast in LR white resin[4] (Table 2.2). Once cast, the micro sections (~150-200μm thick) were cut using the Exakt Diamond edge cutting saw[5] (Type 310CP) using a 0.3mm thick blade. Technovit Glue was used to stick the thick sections to a Perspex slide (~ 4 mm thick). Thin sections (~70μm thick) were prepared using an Exakt micro grinding system[5] through four regions of the femur: the proximal, mid and tip of the femoral component region and one distal to the implant tip (Figure 2.3). Sections were polished on the Motopol 2000 system[6] with polishing cloth and 0.5μm Alumina polishing solution. Once prepared, the sections were stained with Toluidine Blue (to stain cell nuclei) for 20 minutes and Paragon (to stain new bone) for 25 minutes. To preserve the slides, cover slips were placed over the section using a Pertex mounting medium.

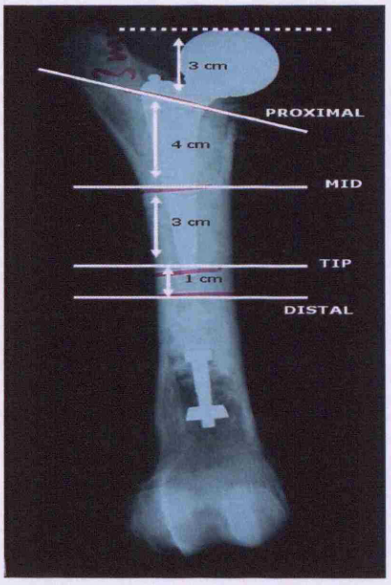


Figure 2.3: A radiograph demonstrating where each femoral implant was sectioned for histological analysis.

[3] BDH laboratory ; [4] Agar Scientific Ltd
[5] Exact, Apparateban GMBH Robert-Koch-Strusse 5, D-22852 Nordestedt; [6] Buehler Ltd

Process	Number of days
10% buffered formal saline	7
50% IMS 50% distilled water	3
70% IMS 30% distilled water	3
100% IMS (repeat step 3 times)	3
Chloroform (repeat Step twice)	1
100% IMS (change IMS twice per day)	3
50% LR white resin 50% IMS	7
100% LR white resin (under vacuum)	14
Cast in LR white resin (1 drop accelerator/10ml resin)	

Table 2.2: Histology Processing Protocol

Images of each thin section were captured using a CCD digital camera and processed using the KS300 Image Analysis Software Package (Imaging Associates, Zeiss, UK). Using the lesser trochanter as a guide, four quadrants were measured in each section (Fig 2.3). Within these quadrants, the following parameters were calculated (using x4 objective lens):

- Cement mantle thickness (at 8 equidistant sites) (Fig 2.4)
- Area of ApaPore (Fig 2.5)
- Area of new bone (Fig 2.5)
- ApaPore-bone contact (Fig 2.6)

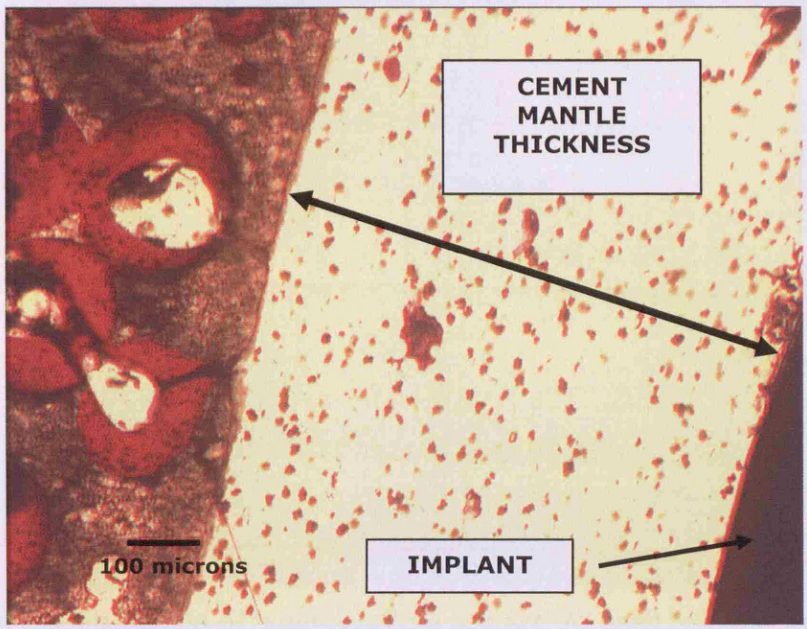


Figure 2.4: Calculation of cement mantle thickness using the KS300 software.

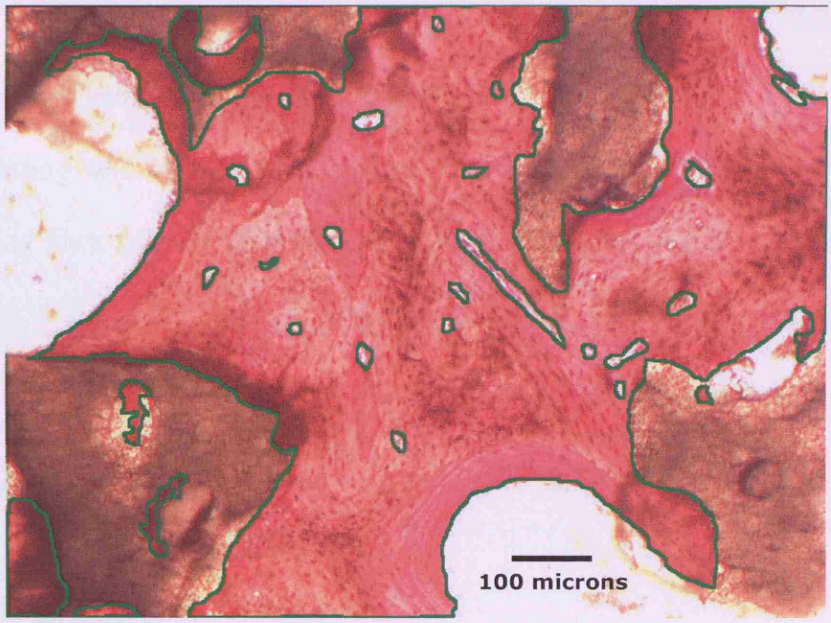


Figure 2.5: Calculation of area of new bone formation and remaining ApaPore. Only areas as demarcated by the green line were measured. All images were analysed on x4 magnification where the full screen size was 1.4mm^2.

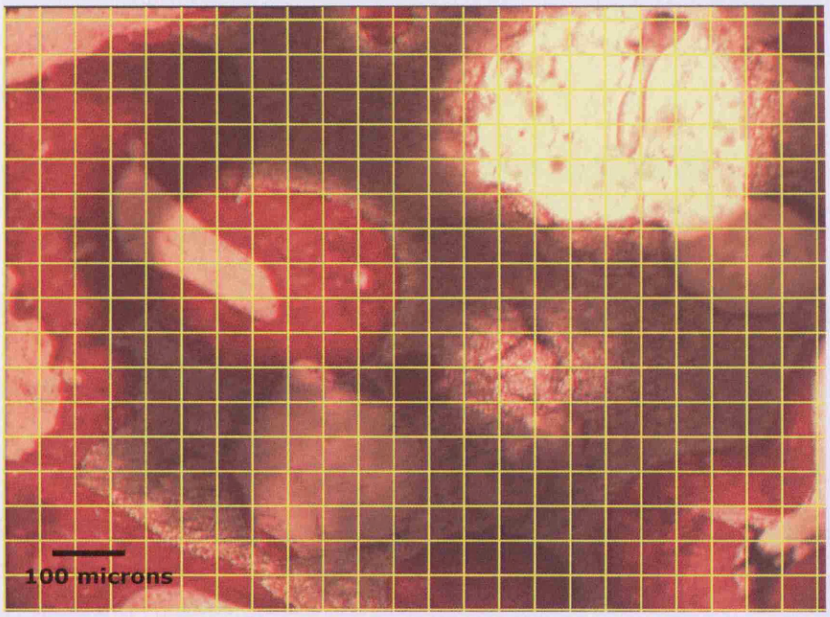


Figure 2.6: Quantification of bone-ApaPore contact. At each relevant intersection on the grid it was noted whether bone or fibrous tissue was in contact with the ApaPore surface.

2.2j. Scanning Electron Microscopy (SEM)

Prior to staining, thin sections were sputter-coated with a layer of gold palladium and viewed using back scattered electron microscopy in a scanning electron microscope (Jeol 5500LV scanning electron microscope (Zeiss, Germany)). Only the distal sections from each animal in each experimental group were assessed using this technique.

2.2k. Statistics

All data collected from the study was analysed using the SPSS (v10.1) statistical software package. A Kolmogorov-Smirnov test revealed the data to be non-parametric and the Mann Whitney U test was used to compare results between the different experimental groups. The significance level was set at $p \leq 0.05$ in all tests.

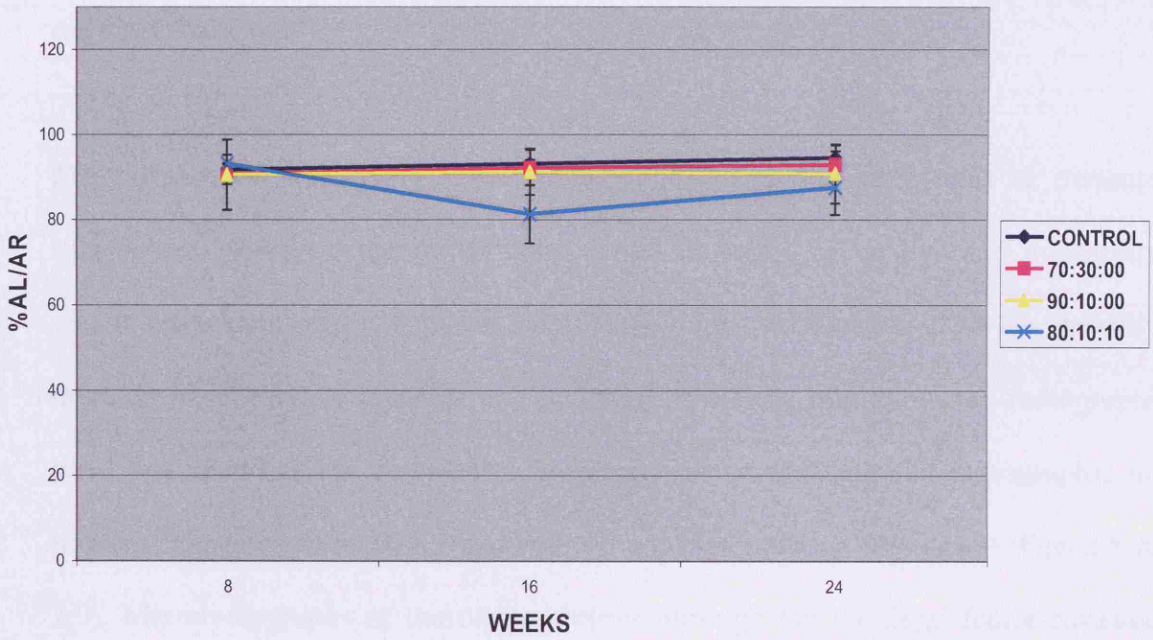
2.3. Results

2.3a. Ground Reaction Force

All pre-operative results were normalised to the average %AR/AL (mean: 100.82 ± 0.63). There was no significant difference in GRF values, when each of the four experimental groups were compared with each other preoperatively or at 8, 16 and 24 weeks post surgery. However, there was a significant decrease in limb function in Groups 1, 2 and 4 compared with preoperative values at 8 and 16 post-surgery, but this had improved by 24 weeks (Table 2.3, Fig 2.7). Function was not significantly affected in Group 3 at any time point.

Figure 2.7

A COMPARISON OF GRF DATA COLLECTED AT 8, 16 AND 24 WEEKS POST-SURGERY (Values are Normalised)



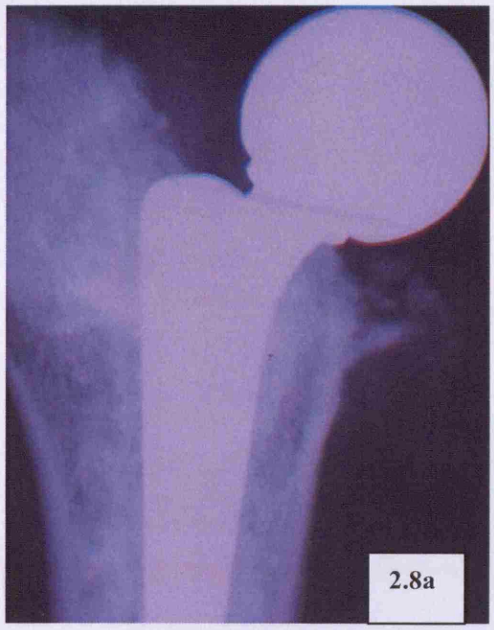
Group	16 week results		24 week results	
	Mean	p value	mean	p value
1	93.09 ±3.33	0.021	94.55 ±2.99	0.035
2	92.14 ±1.87	0.001	92.93 ±1.87	0.006
4	81.30 ±6.81	0.001	87.53 ±6.33	0.021

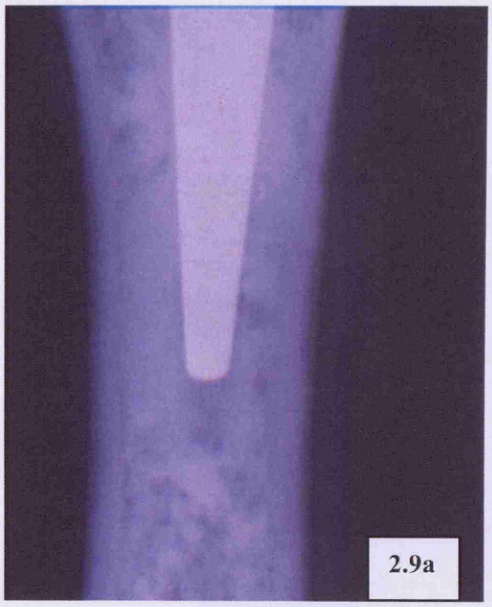
Table 2.3: GRF (mean) at 16 and 24 weeks post surgery compared with pre-operative values. The significance level was set at $p \leq 0.05$.

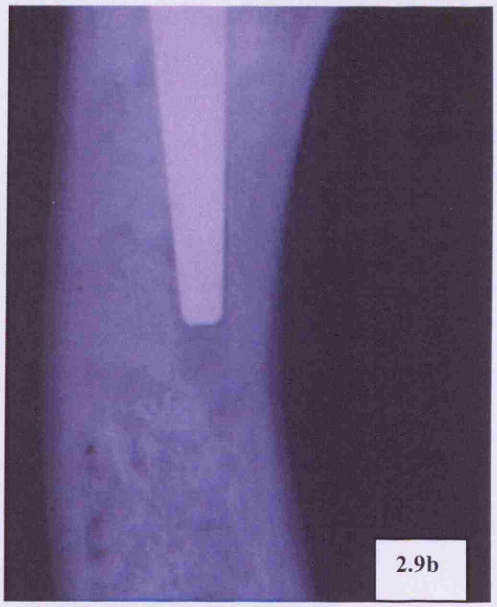
2.3b. Radiography

Anterior-Posterior (AP) and medial-lateral radiographs of retrieved femora demonstrated that all implants were surrounded by densely impacted bone graft substitute. This is important because if the graft material did not surround the stem, the model may be compromised as the cement could integrate with host bone and not the graft.

The purpose of performing serial radiographs during the study was to measure subsidence. However, the radiographs varied in terms of quality and uniformity which made comparison between serial radiographs difficult. Due to the inability therefore to accurately measure any potential prosthesis migration, the radiographs were not analysed for subsidence. However, on scrutinising the radiographs, all implants appeared to be well fixed with no obvious signs of subsidence (Figs 2.8 & 2.9). Microradiographs of transverse sections through the proximal femur revealed impacted graft material surrounding the stem and bone cement (Figure 2.10). Similar radiographs distal to the stem tip showed the intramedullary cavity within the tubular cortical bone to be packed with graft material (Fig 2.11). There was no evidence of cement penetration into the graft at this level.







Figures 2.8 & 2.9: (a) Anterior-Posterior and (b) medial-lateral radiographs of proximal and distal femur 6 months following insertion of 70:30 ApaPore: allograft mixture.

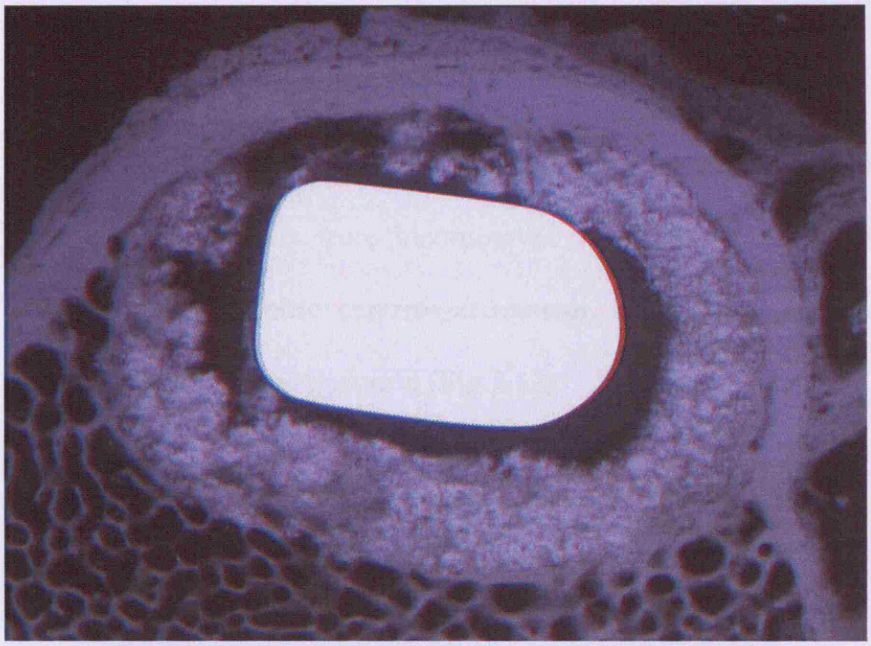


Figure 2.10: Microradiograph of a transverse section through the proximal femur demonstrating impacted graft material (80:10:10) surrounding the stem and bone cement.

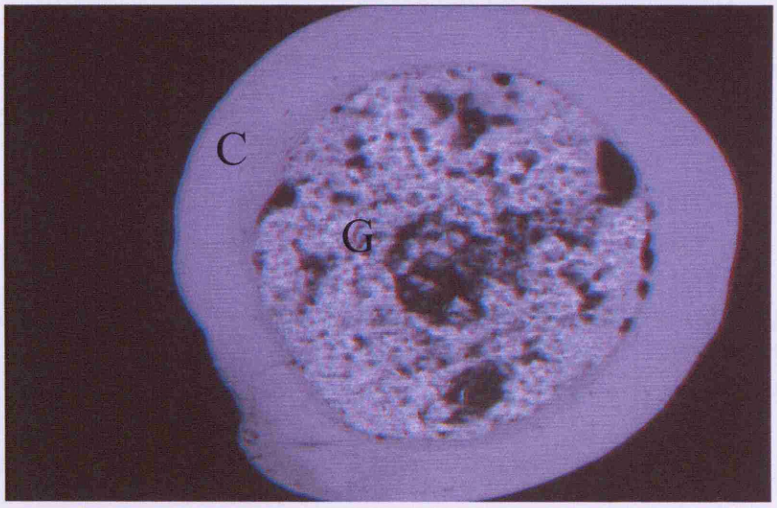


Figure 2.11: Microradiograph in the region distal to the stem tip showing impacted graft material (90:10). Intramedullary cavity surrounded by the tubular cortical bone (C) is filled with graft (G).

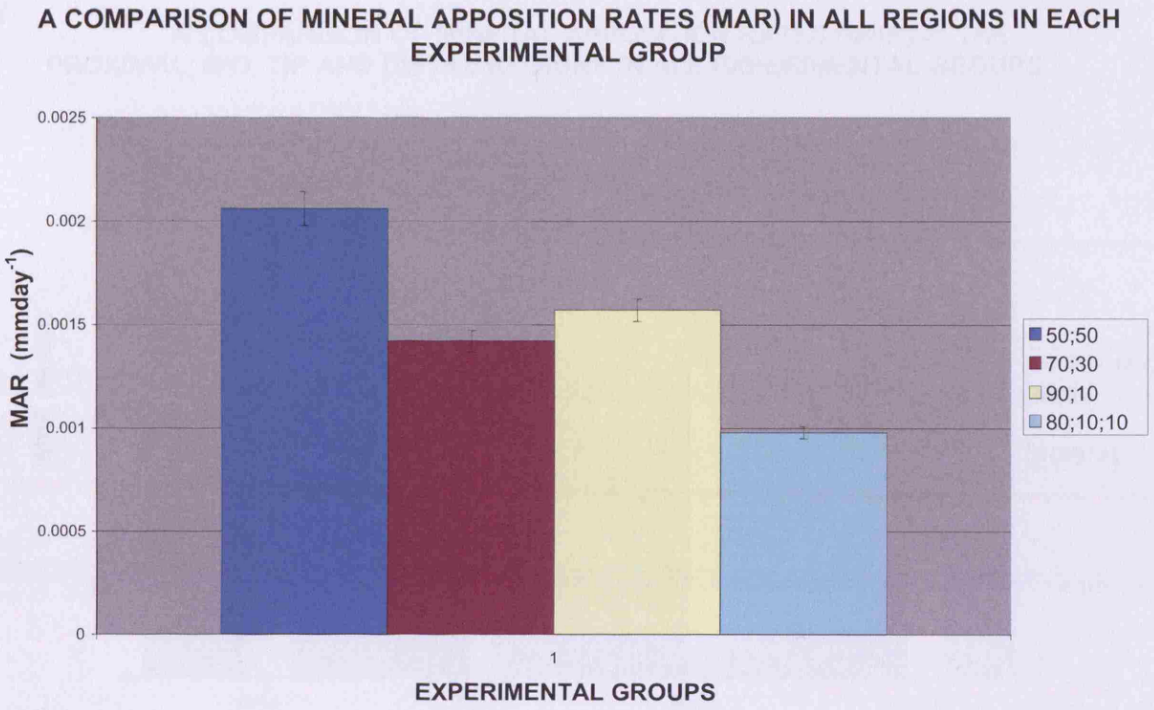
2.3c. Fluorescent Labelling

Mineral apposition rates were calculated in $mmday^{-1}$ and values were compared between the four experimental groups. Cement lines were evident separating newer areas of bone and the markers were incorporated into both remodelled Haversian systems, in a regular concentric centrifugal manner, and in areas of bone where layered appositional growth had occurred (Fig 2.12).

Figure 2.12: Fluorescent labelling demonstrating cement lines separating new areas of bone formation.

Qualitative observation demonstrated that in each of the treatment groups, mineral apposition was greatest at the periphery, at the endosteal surface of bone, and least towards the cement surface. Results from fluorescent markers within the graft material demonstrated significantly greater bone formation rates in Group 1 compared with the other three groups (Fig 2.13). There was significantly less bone formation in Group 4 compared with the other three groups (Table 2.4). However, variations in mineral apposition rates were noted within the different regions of the femur. There was significantly high mineral apposition rates seen in the distal region in Group 1 compared with group 2, but not any other group (Fig 2.14). No significant differences were observed in any of the other regions when the four groups were compared.

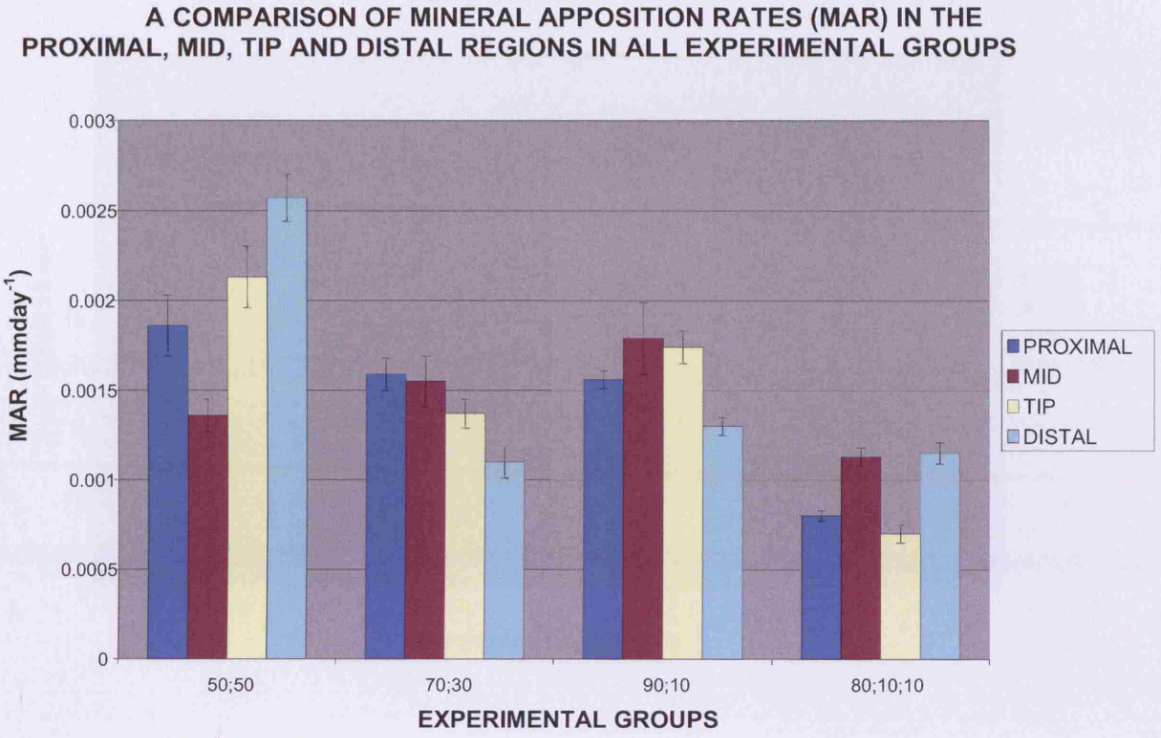
Figure 2.13



EXPERIMENTAL GROUPS	50:50	70:30	90:10	80:10:10
50:50				
70:30	p<0.05			
90:10	p<0.05	0.108		
80:10:10	p<0.05	P<0.05	p<0.05	

Table 2.4: Comparison of mineral apposition rates between the four treatment groups (p<0.05 considered to be statistically significant).

Figure 2.14



2.3d. Cement Mantle Thickness

There was no significant difference in cement mantle thickness amongst the four experimental groups or when the same region of the femur was compared within the four groups (Fig 2.15). However, a significantly thinner cement mantle was noted in the proximal regions of all the groups ($p<0.05$) when compared with the 'mid' region where the thickest cement mantle was identified, except in Group 2 (Fig 2.16).

Figure 2.15

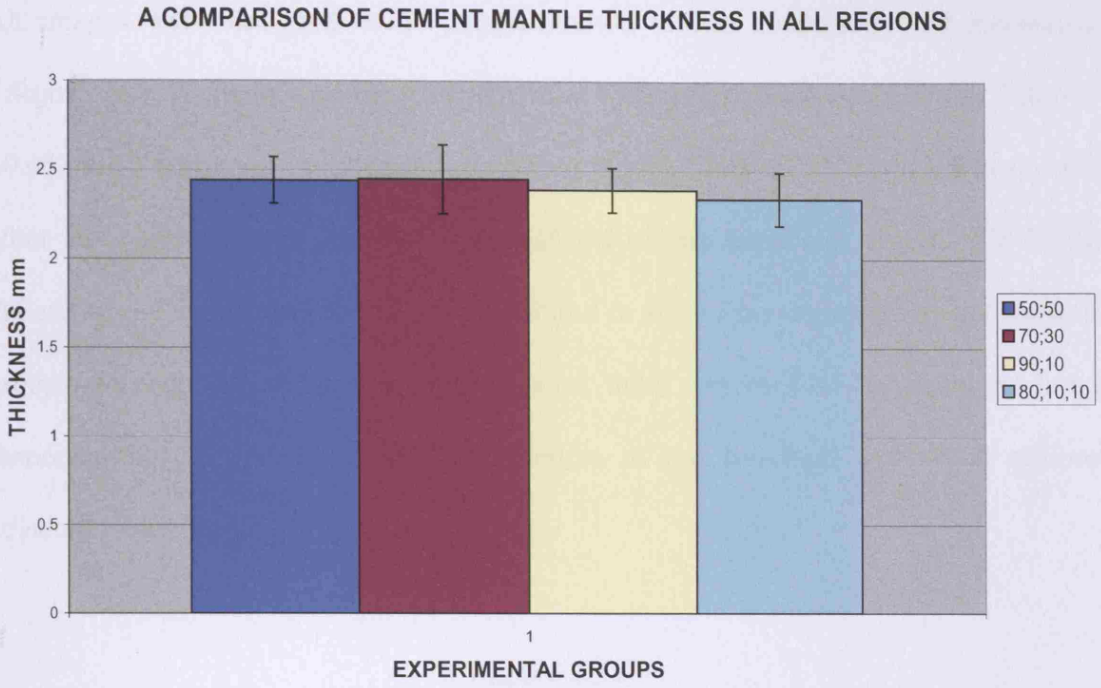
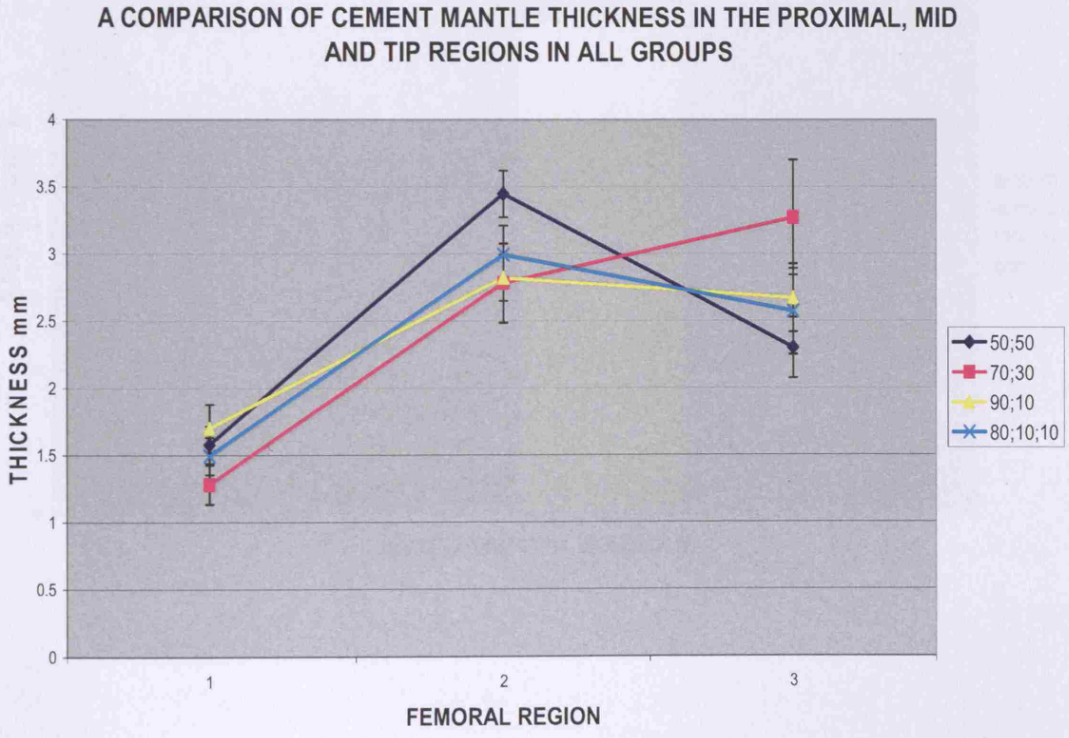


Figure 2.16



2.3e. Area of HA Granules

All images were analysed on x4 magnification within a defined field measuring 1.4mm^2. Overall, there was significantly more bone graft substitute in Group 3 (0.683 ±0.052mm^2) compared with Group 4 (80:10:10) (0.5446 ±0.0224mm^2), but not the other three groups, and this was not confined to any particular region (Fig 2.17). Otherwise, no significant difference was found in any of the regions amongst the four groups. In each of the experimental groups, there appeared to be a general trend demonstrating the presence of HA granules in the proximal and distal regions adjacent to the implant (Fig 2.18).

Figure 2.17

AREA OF BONE SUBSTITUTE IN ALL REGIONS IN ALL GROUPS

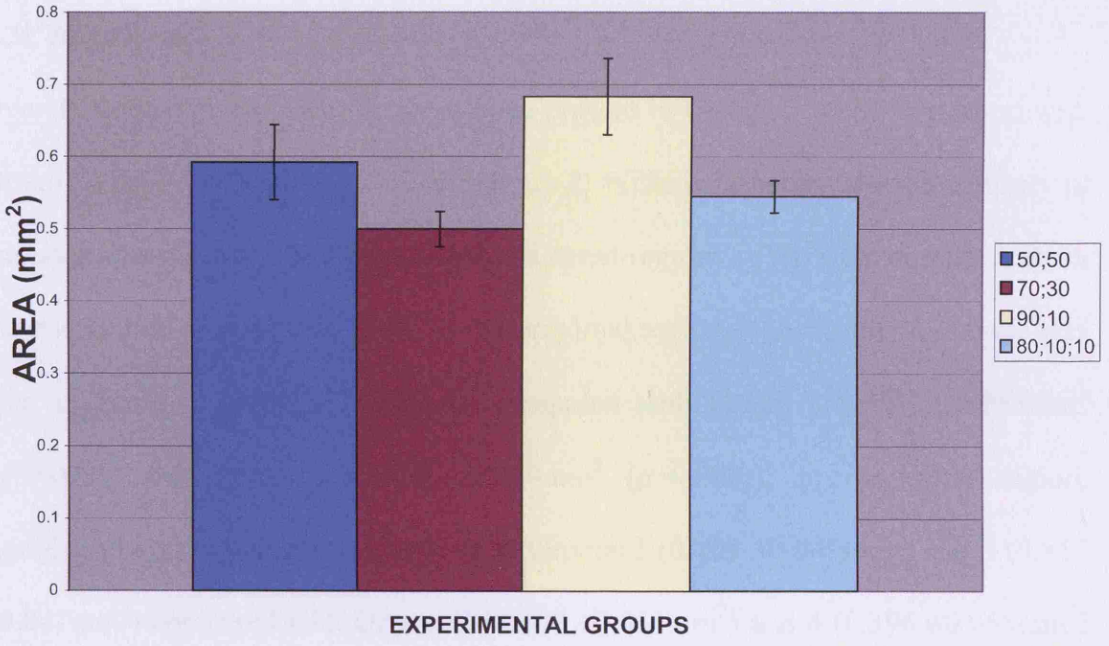
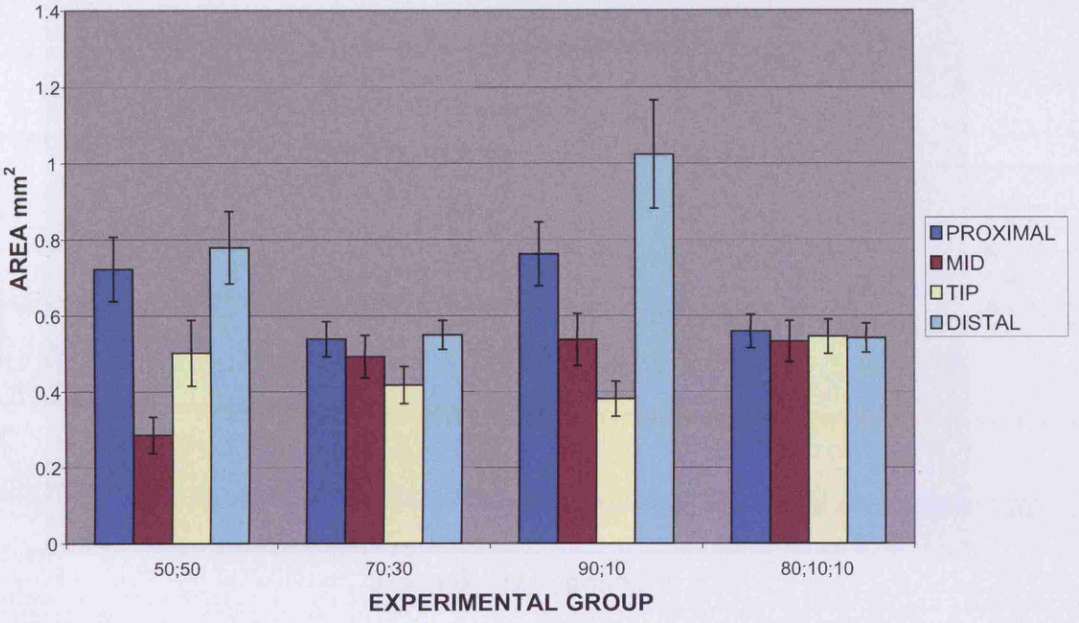


Figure 2.18

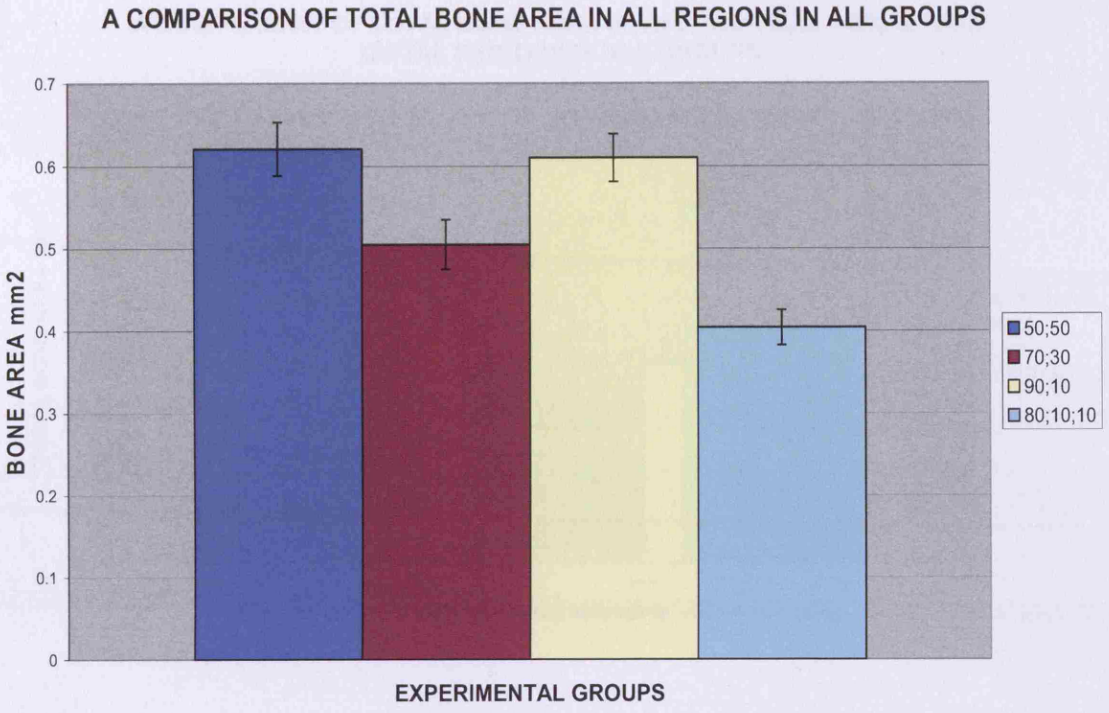
A COMPARISON OF BONE SUBSTITUTE AREA IN THE PROXIMAL, MID, TIP AND DISTAL REGIONS IN ALL GROUPS



2.3f. Bone Area

Overall, there was significantly more bone present in Groups 1 and 3 compared with Group 4 (Fig 2.19, Table 2.5) but not Group 2. Within all the groups, the majority of the bone was observed in the proximal and distal regions of the stem and the least in the mid and tip regions (Fig 2.20). In the proximal region, significantly less bone was seen in Group 4 (0.449 $\pm$0.040mm^2) compared with Group 2 (0.652 $\pm$0.053mm^2 (p=0.003)) and Group 3 (0.713 $\pm$0.059mm^2 (p=0.003)). In the distal region, significantly more bone was quantified in Groups 1 (0.703 $\pm$0.049mm^2) and 3 (0.817 $\pm$0.047mm^2) compared with Groups 2 (0.407 $\pm$0.053mm^2) and 4 (0.396 $\pm$0.055mm^2) (Table 2.6). In the mid and tip areas, there was no significant difference in bone area in any of the groups.

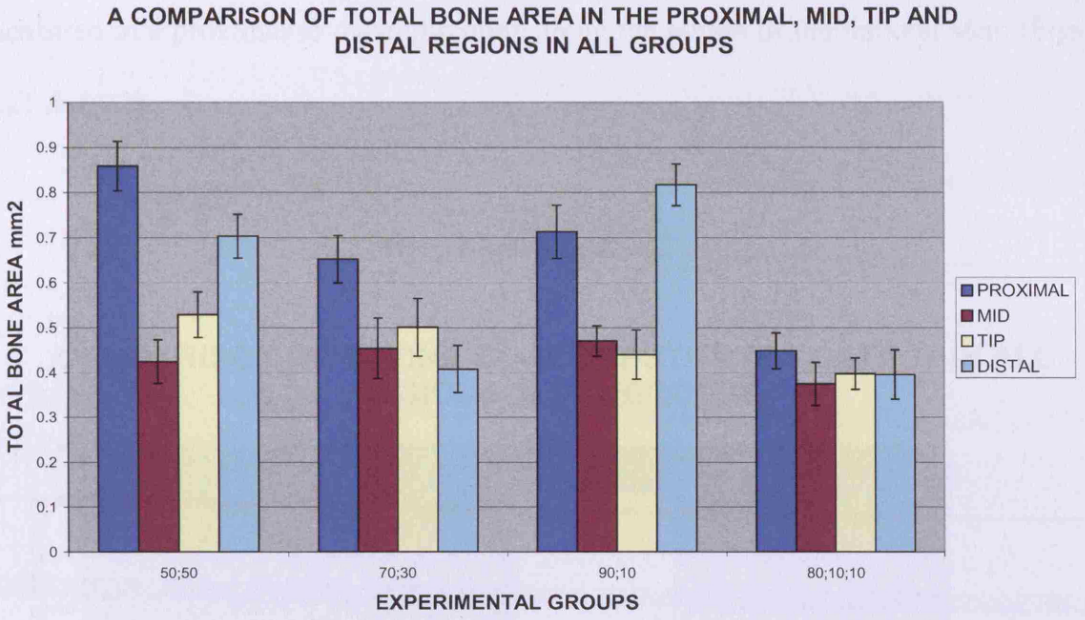
Figure 2.19



EXPERIMENTAL GROUPS	50:50	70:30	90:10	80:10:10
50:50				
70:30	0.18			
90:10	0.732	0.42		
80:10:10	P<0.05	P<0.05	P<0.05	

Table 2.5: Comparison of amount of bone within the graft between the four groups

Figure 2.20



EXPERIMENTAL GROUPS	50:50	70:30	90:10	80:10:10
50:50				
70:30	0.002			
90:10	0.167	P<0.05		
80:10:10	0.001	0.757	P<0.05	

Table 2.6: Comparison of bone presence in the distal stem amongst the 4 groups

2.3g. Bone-Bone Substitute Contact

There was significantly increased bone-bone substitute contact in Groups 1 (82.592% ±1.053) and 3 (81.534% ±1.571) compared with Groups 2 (61.716% ±3.268) and 4 (66.662% ±2.329). However, no significant differences were identified when Groups 1 and 3 or Groups 2 and 4 were compared. Similarly, no significant difference was

demonstrated within any of the groups when %Bone-Bone substitute contact was measured in a proximal to distal direction along the length of the femoral stem (Figs 2.21 & 2.22).

Figure 2.21

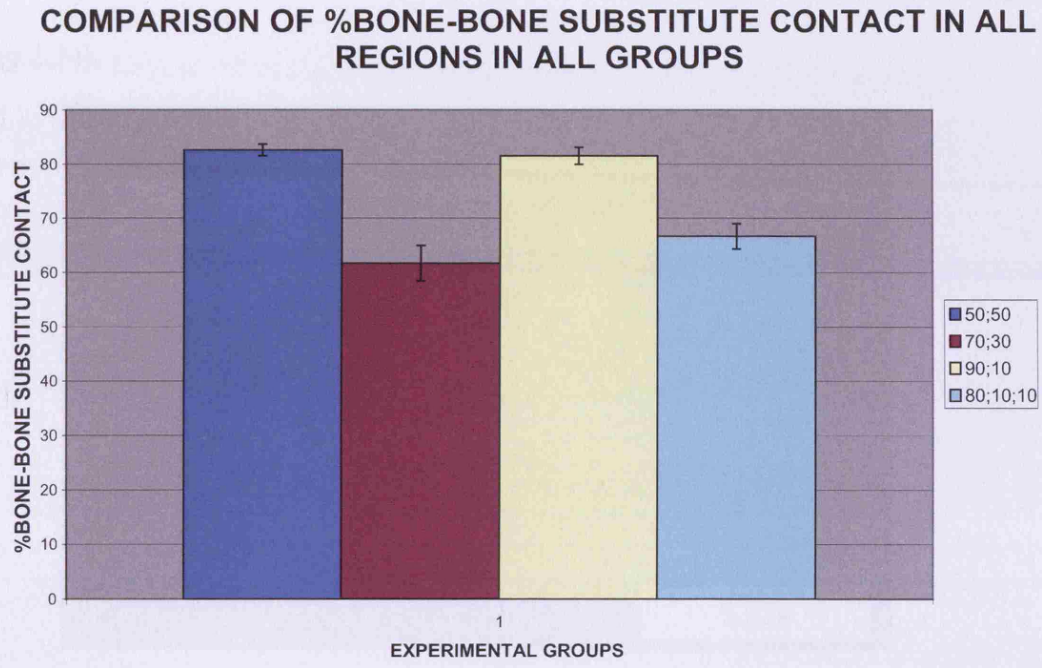
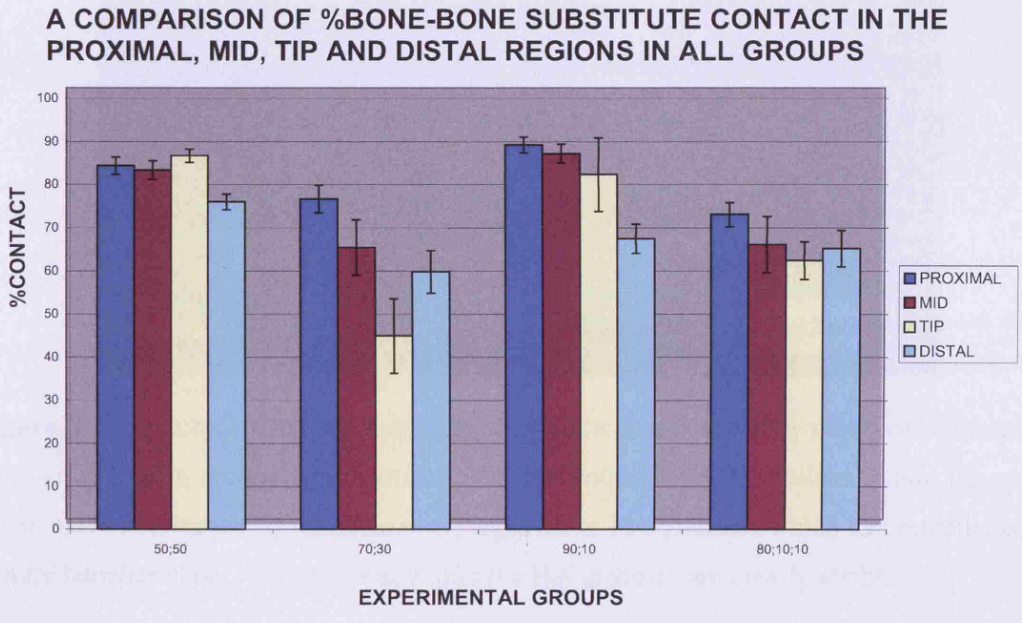


Figure 2.22



2.3h. Backscattered Scanning Electron Microscopy

Backscattered scanning electron microscopy identified bone growth from the endosteal surface of the bone into and within HA granules within the medullary canal (Fig 2.23). Haversian systems were observed adjacent to HA granules which had remodelled within mature lamellar bone. In addition, in all groups, bone was identified in contact with the outer surface of the HA granules and also within pores (Fig 2.24).

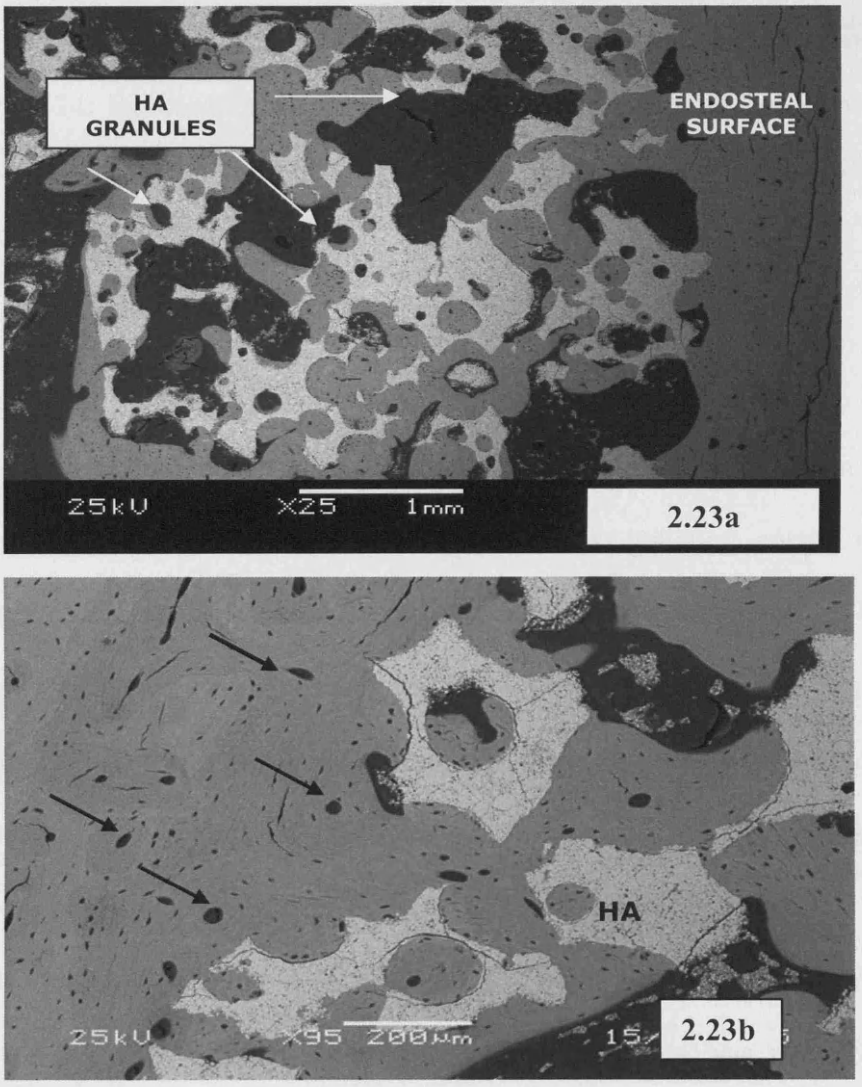


Figure 2.23: Backscattered scanning electron microscopy showing new bone formation: (a) bone growth from the endosteal surface into and within the HA granules within the medullary canal and (b) Haversian systems *(arrows)* adjacent to HA granules which had remodelled within mature lamellar bone. Fracture lines within the HA granules are clearly visible.

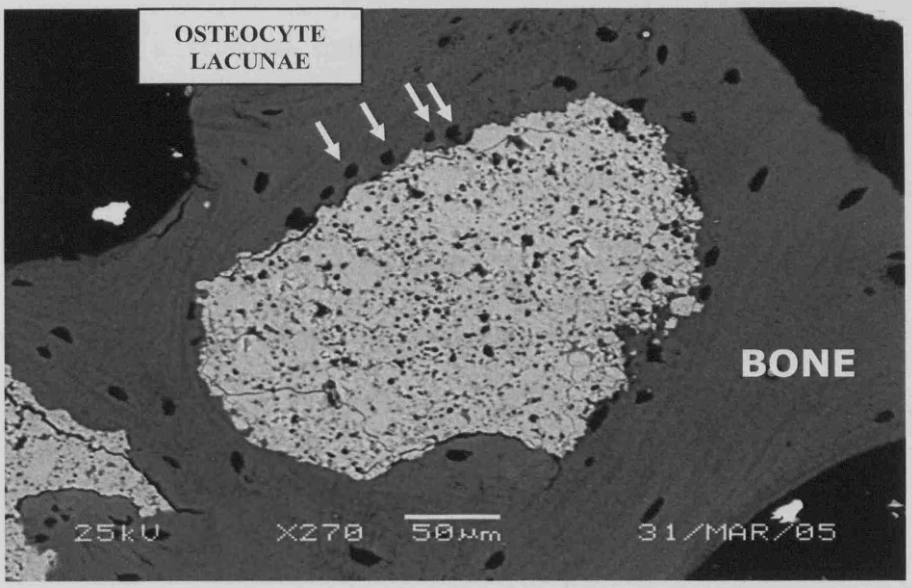


Figure 2.24: Backscattered scanning electron microscopy images showing bone in contact with the outer surface of HA granules and also within pores.

2.3i. Histology

Histology showed complete reorganisation of the intramedullary canal. The implant stem was surrounded by a layer of cement, which in turn was surrounded by HA granules around which new bone had formed. The existing cortex was evident. Histological analysis of specimens in all groups demonstrated abundant bone growth in direct contact with ApaPore/Actifuse and forming a network of mature lamellar bone adjacent to the implant and within the medullary cavity (Figs 2.25 & 2.26). Broken particles of HA were also seen, but were embedded within bone. In many instances, bone lamellae within pores were centripetally organised. Blood vessels were also observed within the pores and were often located centrally and surrounded circumferentially by lamellar bone. The cement interface was inter-digitated with both bone and HA particles. At this interface, HA granules were seen either in direct contact with cement or separated from it by a thin layer of bone. Small amounts of allograft placed adjacent to the implant during surgery could be identified in localised

areas, providing a scaffold onto which new bone grew (Figs 2.26 & 2.27). Histological examination demonstrated fragmentation of HA granules in some specimens within each of the experimental groups. In all specimens where fragmentation had occurred, an inflammatory-like reaction with densely stained mononuclear, multinucleated and macrophage-like cells were present, along with a fibrous tissue interface separating HA granules from the bony surface (Figs 2.28 & 2.29). Sub-micron sized particles had been engulfed by macrophages and foreign giant cells.

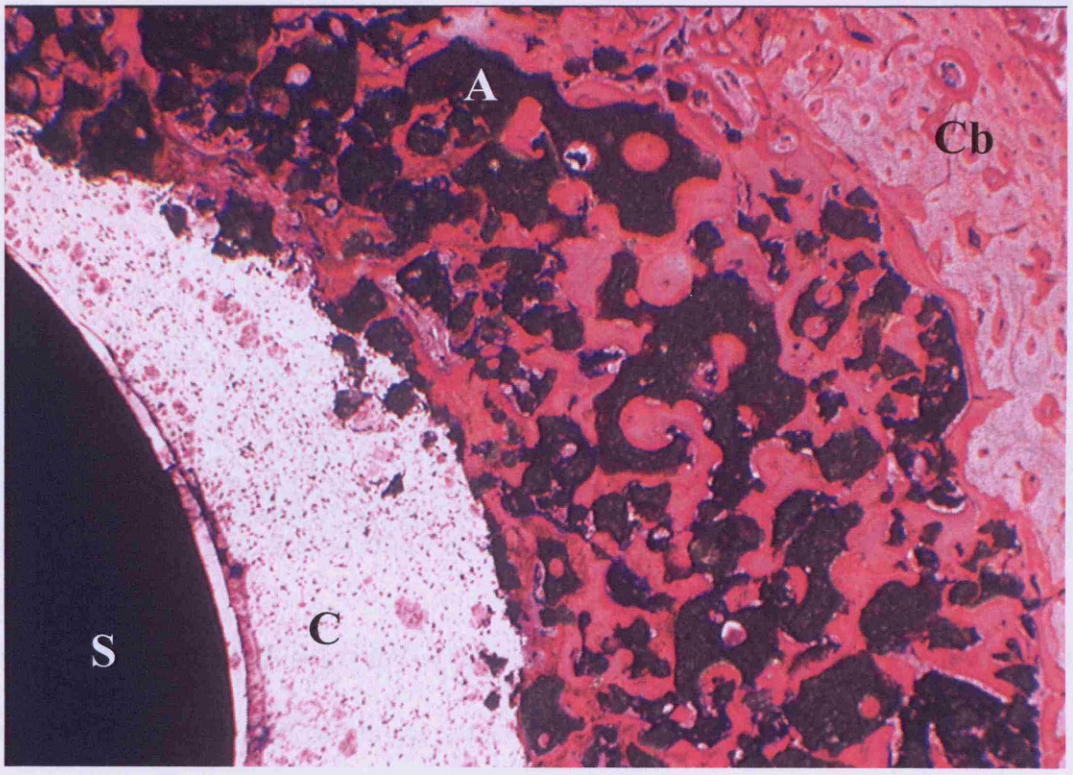


Figure 2.25: Histological analysis of specimens (low power): photomicrograph showing stem (S) cement (C) and cortical bone (Cb) with new intramedullary bone formation (B) between the HA granules (A)

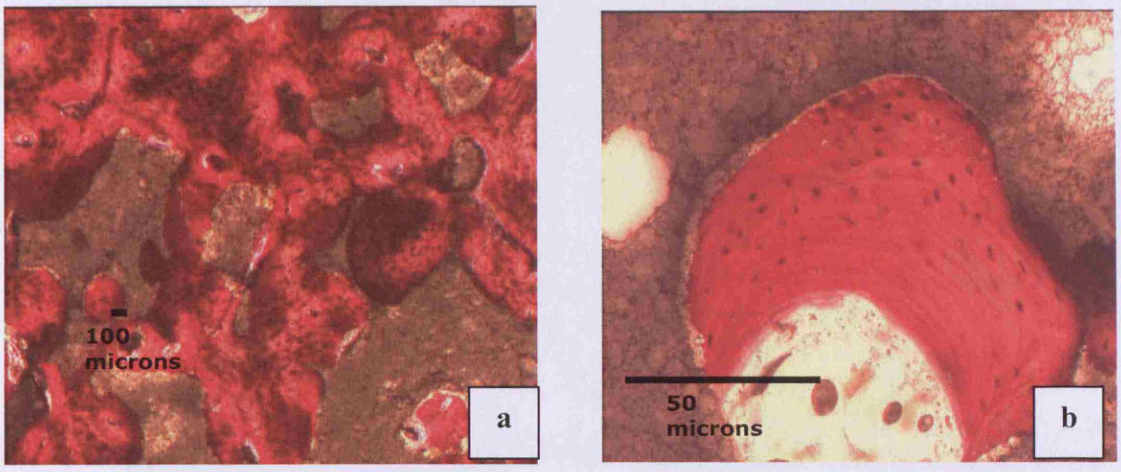
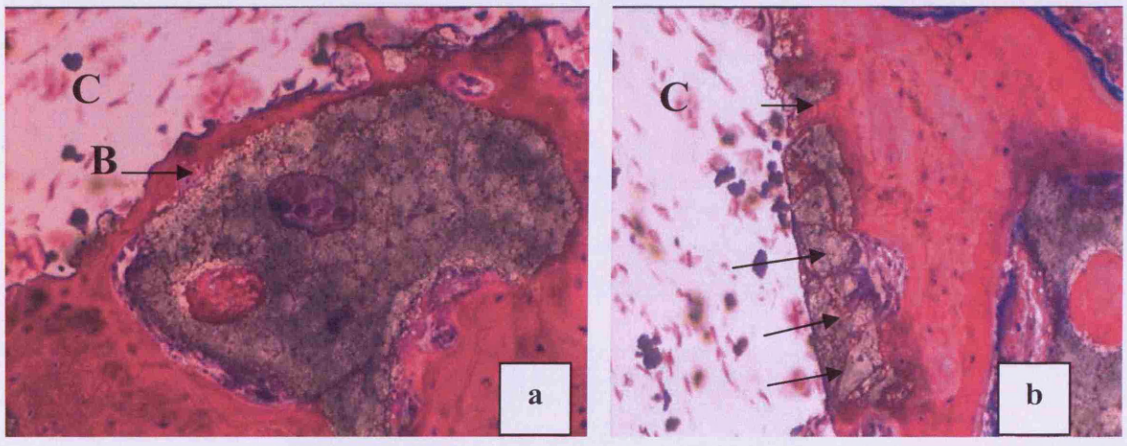


Figure 2.26: A photomicrograph (a) low power and (b) high power demonstrating mature bone surrounding HA granules within the medullary canal.



Figures 2.27: Photomicrographs (a) low power and (b) high power showing the HA-cement interface. HA particles are separated from the cement (C) by a thin layer of bone (B) or are in direct contact with the cement (arrow).

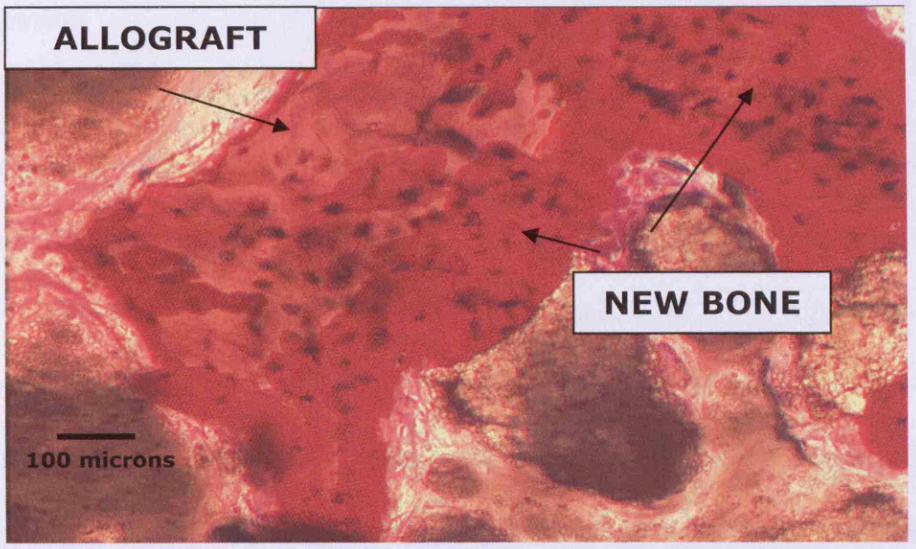
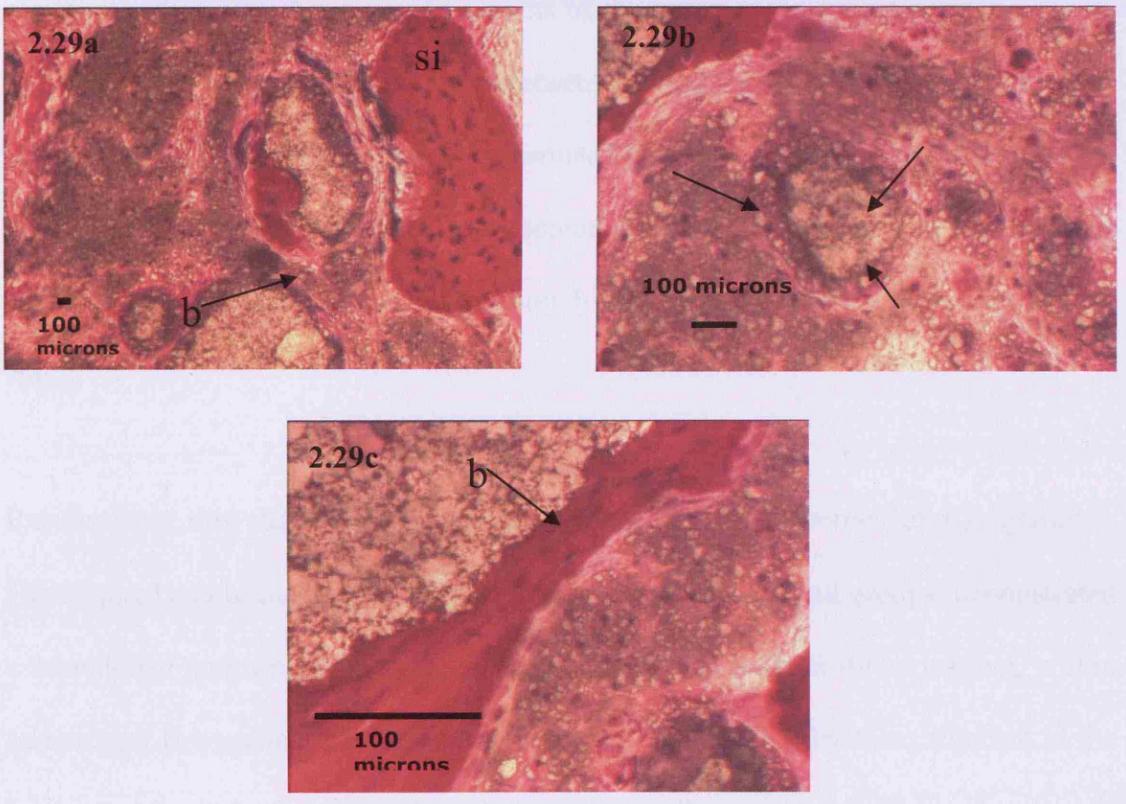


Figure 2.28: Photomicrograph showing allograft and newly formed bone.



Figures 2.29: (a) x20 (b) x40 and (c) x80 magnification micrographs of fragmented HA granules showing how particles of HA are released from around the edges of the HA granules. This produces a histiocytic response where particles are phagocytosed by macrophages. Particle release occurs only where there is a soft tissue interface (si) and not where new bone is in direct contact with the HA granules.

2.4. Discussion

An ovine hemiarthroplasty model was selected for this study because the sheep femur is known to be tubular with minimal cancellous bone and poor blood supply, making it a suitable experimental model which resembles the bone stock encountered in a clinical revision situation (Goel et al., 1982; Eitel et al., 1981). The hip forces in the sheep correlate well with those in the human (Bergmann et al., 1984) and thus, functional loading of the graft-composite would be similar to those encountered in the clinical setting. Furthermore, the primary ovine hip replacement model has previously successfully been used to investigate a revision scenario (Blom et al., 2005; McGee et al., 2004). However, there may be aspects of the tissue environment prior to revision hip surgery, in humans, that are not represented in this model i.e. endosteal erosions, residual inflammatory tissue and compromised host bone quality (Verdonschot et al., 2001). The inability, therefore, to recreate with complete accuracy the tissue environment encountered during revision hip surgery remains a limitation of this study.

Results from this study confirmed the osseoconductive properties of HA granules. Histological and backscattered SEM analysis of specimens in all groups demonstrated a remodelled endosteal surface with abundant bone growth in direct contact, within and around HA granules, forming a network of mature lamellar bone adjacent to the implant and within the original medullary cavity. Post-mortem radiographs showed implants surrounded by densely packed graft material and ground reaction force data demonstrated functional hips in all cases. All animals were able to use their operated limb immediately post-surgery and during the course of the experiment. Although there was an initial decrease in limb function in Groups 1, 2 and 4, this improved by

24 weeks. Function in Group 3 (90:10) was not significantly affected at any time point. GRF results are a measure of implant function and how well the animal used its operated limb and a persistent reduction in function can be evidence of implant loosening.

Analysis of cement mantle thickness did not reveal any significant difference amongst the tested groups. Cement mantle was thickest in all groups (except Group 2) in the 'mid' region of the femoral component when compared with the proximal and tip regions. Cement mantle was not observed distal to the stem tip in any of the groups. These results are supported by a recent study (Frei et al., 2004) which investigated the graft–cement–host bone interface after impaction allografting of a femoral component in human cadaveric femora and showed significantly increased cement mantle thickness in the 'mid' region, with absence of cement mantle distal to the stem tip. The study concluded that although cement-endosteal surface contact enhanced the allograft-cement composite–host bone interface strength, it was significantly weaker compared to a primary THR. The authors suggested that the host bone interface may be enhanced by postoperative allograft remodelling, which may be the key to the success of the impaction allografting technique.

In all groups, the cement interface was inter-digitated with both bone and HA particles. At this interface, HA granules were seen either in direct contact with cement or separated from it by a thin layer of bone. Bone particles within the cement mantle may give rise to cement or bone-cement composite fractures which have been associated with subsidence of the stem (Masterson et al., 1997). However, Dai et al. (1991) have shown that bone particles embedded in cement can be replaced by new

bone and form a viable cement-bone interface. In that study the strength of this viable interface was significantly higher than cement alone, but it is not known whether these findings are applicable to impaction grafting.

The administration of fluorescent bone markers allowed quantification of mineral apposition rates and results demonstrated significantly greater bone formation in Group 1 (50:50) compared with the other groups. Other authors have supported similar findings (Moore et al., 1987; Virolainen et al., 1997). A study by (Pratt et al., 2002) comparing impaction grafting with allograft and allograft-80%: HA-20% TCP showed larger quantities of new bone within the allograft only group. However, in this study we have shown that allograft mixed with up to 90% HA has similar integration and osseoconductive properties as allogenic bone and this has significant clinical implications in terms of allograft savings.

The bioactivity of hydroxyapatite has been shown to be significantly enhanced with the incorporation of silicate ions into the hydroxyapatite structure (Gibson et al., 1999). Furthermore, silicate-substituted hydroxyapatite has been demonstrated to enhance osteoblastic differentiation and stimulate new bone formation compared with phase pure hydroxyapatite (Patel et al., 2002; Hing et al., 2006). However, in this study mineral apposition rate and new bone formation was significantly lower in Group 4 (80:10:10) compared with Group 3 (90:10). It therefore appears that the impaction process alters the mechanical and biological properties of silicate-substituted hydroxyapatite differently to that of phase pure hydroxyapatite. It may be that the Actifuse particles crunch under impaction or deform elastically and are

damaged to a significant level. In-vitro studies evaluating the time dependent mechanical properties of Actifuse would provide answers to this question.

To minimise the disparity in impaction grade, all specimens were prepared and manually impacted by the same person. There may be discrepancies in the impaction grade between the groups due to the effects of variable particle size distribution and the reduced compressibility of hydroxyapatite compared to allograft on the level of impaction (Verdonschot et al., 2001). Deformation and intergranular motion can occur during compaction which can alter the impaction grade. Verdonschot et al., (2001) report that the particle size and porosity are important factors in total deformation, with less deformation exhibited in the biomaterial particles than in human bone graft. The degree of compaction of the graft is vital to the likelihood of bone incorporation and stability of the implant and will determine the strain of the graft (Malkani et al., 1996). Masterson et al. (1999) stated that the level of impaction is more important than stem design to the success of an implant. Only after adequate impaction is the morsellised graft strong enough to carry load (Masterson et al., 1997). Hydroxyapatite, when manufactured in particulate form, can be harder than morsellised cancellous bone. Although this may affect the ease with which HA can be impacted, ultimately this will improve compaction and enhance implant stability. Pratt et al (2002) have discussed the possibility that better impaction and compaction of the graft and the resultant cohesion may compromise neovascularisation and incorporation of the graft; adequate vascular supply is an essential factor in osseointegration. A lower impaction grade may allow and encourage ingrowth of vessels and new bone. A firm impaction technique is crucial in achieving maximal graft-host bone contact. An advantage of using bone substitutes like ApaPore is that,

providing that the granules do not collapse, the porosity is engineered in. Histology from our study showed that although there was evidence of fracture of the granules, there were also numerous pores through which blood vessels had penetrated. Increased microporosity of scaffolds has been shown to elicit rapid neovascularisation, essential for osseointegration (Virolainen et al., 1997). Allografts possess only limited osteoinductive capacity (Vaccaro, 2002) and as this study has shown, this does not appear to be that important as the different groups in this study gave similar results.

The biological and mechanical response of biomaterials, including HA, may be affected by particle size and porosity (Verdonschot et al., 2001). Our study utilised HA granules with 60% porosity. Further studies looking at the different available porosities of HA (70% and 80%) with variations in particle size would provide a greater insight into how these variables affect the biological and mechanical response.

A study by Buckland & Lawes (2004) comparing the use of allograft alone with 50:50:Allograft in impaction grafting demonstrated that the 50:50 group had 50% greater mechanical stability, 50% decrease in subsidence and a significant reduction in the variability of the mechanical properties of the graft material. Our study has shown that similar results can be achieved with up to 90% ApaPore. Given these encouraging results impaction grafting using 100% HA granules can be investigated.

6. Conclusion

- Results demonstrated a successful ovine hemiarthroplasty model for hip impaction with well fixed implants observed 6 months post surgery with no obvious signs of loosening or subsidence.

- Hips maintained functional stability throughout when higher amounts of HA mixture were used, with no significant differences identified between experimental groups when limb function was assessed and compared.

- Significantly higher mineral apposition rates were demonstrated in Group 1 (50:50) and significantly lower values in Group 4 (80:10:10). This may be due to the impaction process altering the mechanical and biological properties of Actifuse.

- No significant differences between any of the experimental groups investigated were identified when cement mantle thickness data was compared. In all groups except group 2 (70:30), the thickest cement mantle was observed in the 'mid' region of the femoral component.

- No significant differences in the area of HA granules were identified in any of the groups, except between Groups 3 (90:10) and 4 (80:10:10). Generally in all groups, HA area was increased in the proximal and distal regions of the stem.

- Significantly increased bone area was measured in Groups 1 (50:50) and 3 (90:10) compared with Groups 2 (70:30) and 4 (80:10:10). No significant difference was identified between groups 1 and 3. However, significantly less bone was measured in Group 4. Overall in all groups, bone area was higher in the proximal and distal regions of the stem.

- Results from this study have clearly shown HA granules to be a suitable bone substitute to augment allograft and perhaps replace it in impaction grafting of a femoral component. Conversely, the use of Actifuse in impaction grafting cannot be advocated at the present time. However, a silicon substituted ApaPore or 60% porous Actifuse could be more biologically active than the Actifuse used in this study and this theory warrants further investigation.

CHAPTER 3

THE EFFECT OF MESENCHYMAL STEM CELLS ON THE INCORPORATION AND REMODELLING OF IMPACTION GRAFTING

3.1a Introduction

As advances in molecular biology are made, there has been a gradual move from a tissue approach to a more cellular approach in providing a more efficient means of reconstituting bone stock. Bone marrow contains a population of cells capable of differentiating into bone, cartilage, muscle, tendon, and other connective tissues. These cells, referred to as mesenchymal stem cells (MSCs), have been isolated from fat, muscle, periosteum and bone marrow (Dennis et al., 1992; Haynesworth et al., 1992). Furthermore, techniques for directing the commitment of MSCs into bone cell lineage have now been developed (Jaiswal et al., 1997; Haynesworth et al., 1992). Although, only about 1 in 100,000 nucleated cells are stem cells in the marrow (Friedenstein et al., 1987), in-vitro expansion over one-billion fold is possible and produces cells without a loss in their osteogenic potential (Bruder et al., 1998). At our Institution, sheep MSCs have successfully been differentiated into adipocytes, chondrocytes and osteoblasts (Kalia et al., 2006) and studies have demonstrated increased bony contact and in-growth to massive endoprostheses when the implant was sprayed with autologous MSCs (Kalia et al., 2006).

One possible approach to solve the problem of reduced bone stock in revision arthroplasty is to implant MSCs directly to the site of the bone defect. The tissue-specific differentiation of MSCs in a controlled manner is vitally dependent on the matrix or local environment in which the MSCs reside (Caplan, 1990). An ideal matrix carrier needs to fulfil certain criteria (Bruder et al., 1998): it should permit loading and retention of MSCs; it should have osteoconductive, osteoinductive and osteogenic properties; it should be made up of materials normally found in the bone-turnover cycle; it should provide immediate strength and support; it should have a

porous structure for stem cells to grow and for blood vessel invasion; it must persist for a sufficient period at the implantation site to maintain and release bioactive cells, but at the same time be biodegradable to prevent any deleterious effects of the residual carrier on new bone formation.

Hydroxyapatite (HA) has been shown to be a good scaffold vehicle because of its porosity, high biocompatibility and osteoconductivity (Vaccaro, 2002; Harris and Cooper, 2004; Cooper et al., 2001). Allograft has osteoinductive and osteoconductive potential and is an excellent scaffold. Both matrices support bone formation by acting as a scaffold for angiogenesis, cell recruitment, and ultimately osteogenesis by host cells (Cornell and Lane, 1998). Thus, a composite graft can be formed from the osteoconductive matrix of an allograft and/or HA combined with mesenchymal stem cells (MSCs), which provide osteoinductive and osteogenic properties. In this way, the allograft and/or HA acts as a delivery system and scaffold for the bioactive MSCs, allowing the passage of osteoblast progenitor cells to the graft site and facilitating bony ingrowth.

HA loaded with MSCs have been reported to have a similar osteogenic potential to autogenous particulate cancellous bone and marrow (Yoshikawa et al., 1998; Boo et al., 2002). MSCs loaded onto a porous hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold have been shown to regenerate bone in a large segmental femoral defect in rats (Kadiyala et al., 1997). By 8 weeks, more than 40% bone fill was achieved compared with only 10% of fill in cell free samples and 17% fill in samples loaded with fresh marrow only. Similar results have been observed with human MSCs, loaded onto a porous HA/TCP cylinder, implanted into osteoperiosteal segmental defects in the femur of adult athymic rats (Bruder et al., 1998).

Furthermore, in the latter study, all of the MSC loaded samples produced uninterrupted bone marrow formation between the two ends of the implant, whereas all the cell-free samples showed bone formation only at the bone/ceramic interface. Similar results reproduced in dogs, has proved that this technology is transferable to larger animals, and that the application of this technique to humans is feasible (Bruder et al., 1998; Kon et al., 2000).

Intra-operative measurement of forces during femoral impaction allografting in revision hip surgery have shown a range of 3 to 9 kN (Phipps et al., 2002) with the majority of the impactions being between 3 to 6 kN. In vitro, stem cells are able to survive impaction forces of up to 6kN with full recovery within eight days (Korda et al., 2006).

3.1b. Hypothesis

The addition of MSC's to allograft or hydroxyapatite by impaction grafting will enhance the amount of new bone formation when compared with impaction of allografts or HA alone in an un-cemented ovine model.

3.1c. Objectives

The objectives of this study were to assess the contribution of stem cells to the material properties of new bone/ graft composite using defect densitometry and histomorphometric analysis.

3.2 Methods

3.2a. Preparation of cancellous allograft and HA

Long bones were removed from healthy sheep that were euthanased as part of an unrelated study. The bones were stored in double-layer polyethylene wrappings at a temperature of -20°C. The graft was prepared in a similar fashion to that described in the previous chapter. HA was derived from 2–5mm diameter calcium phosphate ceramic granules (ApaPore 60™; ApaTech Ltd), with a pore size of 230 μm and porosity of 60%.

3.2b Harvesting of sheep mesenchymal stem cells

Under general anaesthesia, an area of 10 x 10 cm over the iliac crest of the sheep was prepared by shaving and scrubbing. After aseptic preparation, a stab incision with a No 11 scalpel was made through the skin. A Rocket Medical biopsy needle (11 gauge, 10cm) was introduced into the iliac crest and two 4mls aliquots of bone marrow aspirated into 5ml syringes, preloaded with 1 ml heparin (1000 iu/ ml) (Fig 3.1). The marrow aspirates were oscillated gently, transferred into sterile 10 ml containers and placed on ice for immediate transfer to the laboratory.

3.2c Purification and culture of mesenchymal stem cells*

The MSC's were separated using a Ficoll-Paque® density gradient technique similar to that described for the isolation of MSC's in other species (Rickard et al., 1996). The initial 4mls of bone marrow aspirate was layered gently onto 3ml Ficoll (Ficoll Paque PLUS, Amersham Pharmacia Biotech, UK). This layered mixture was then

* Purification, culture and seeding of the MSCs onto the graft was carried out by Miss M. Korda (Cell scientist at the Institute of Orthopaedics, UCL)

centrifuged at 1510 rpm (400 ***g***) for 30 minutes until a straw coloured buffy layer formed in between the plasma and Ficoll erythrocyte residue. This buffy layer was recovered and washed by adding 10ml Dulbecco's Modified Eagles Medium (DMEM)[1]. The sample was spun at 2000rpm (702 ***g***) for 10mins to remove the heparin and Ficoll. The supernatant was discarded and the cell pellet re-suspended in 12 ml DMEM. This cell suspension was then plated into T25 (25-cm^2) flasks. The cells were maintained at 37°C in a fully humidified atmosphere containing 95% air and 5% CO_2.

The primary seeded cells were allowed to adhere to the flask for 2 days before changing the medium and thereafter, the medium was changed every 3 days for 14-16 days when colony-forming units were visible. When culture dishes reached confluence (usually 2 weeks after the original plating), cells were harvested with 0.05% trypsin (Sigma), washed with 5mls of PBS and re-suspended in DMEM. These cells were expanded for a further 5-9 days until confluent.

Cells were removed from the flask using trypsin digestion and centrifuged at 2000rpm (702 ***g***) for 5mins to pellet the cells. The medium supernatant was discarded and the cell pellet re-suspended in 1ml fresh DMEM. The cells were re-plated and the medium changed every 3 days. Cells that had reached passage 2 to 6 were used in this study. A 20 µl aliquot was aspirated and counted in a haemocytometer to give the cell count/ml.

[1] Sigma Aldrich, Poole; 4500mg/l glucose, L-glutamine and sodium pyruvate with 10% foetal calf serum,
penicillin 50 iu/ml and streptomycin 50 µg/ml)

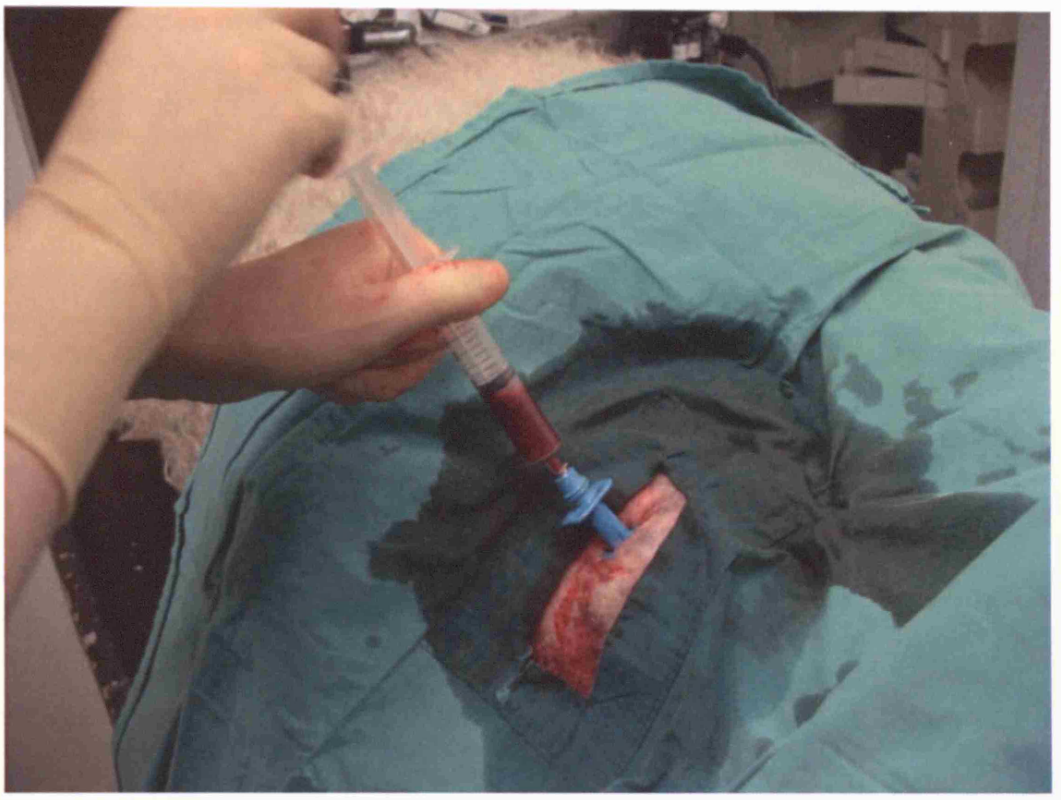


Figure 3.1: Bone marrow harvesting from the iliac crest

3.2d Suspension seeding of MSC's onto graft material*

MSC's were seeded at 2×10^6 cells/g onto 3.5g of allograft or HA. All cells were confluent at passage 3. MSC's were trypsinised from culture flasks and counted. Cells were seeded onto graft material in a universal tube with 7ml DMEM. Cells were attached to the graft material in the universal tube that was rotated on a TAAB rotator (TAAB, UK) for 2.5 hours. The cells were then transferred to a 6 well plate and were maintained in culture for 4 days prior to the operation.

* Purification, culture and seeding of the MSCs onto the graft was carried out by Miss M. Korda (Cell scientist at the Institute of Orthopaedics, UCL)

3.2e Impaction of composite graft

Treatment mixtures comprising of allograft ± MSCs and HA ± MSCs were impacted at 3kN to form a cylindrical pellet immediately before insertion into a metaphyseal defect. A quantity of the mixture was placed within a specially designed impaction hammer (Fig 3.2) and impacted twenty times by a piston driven by a slap hammer. A standard level of twenty impactions was applied throughout the study, as graft compaction has been shown to occur in the first 20 impactions and little change occurs in the dimensions of the graft thereafter (Phipps et al., 2002). The resulting graft measured 21 x 15mm and was retained within the tube in which it had been impacted.

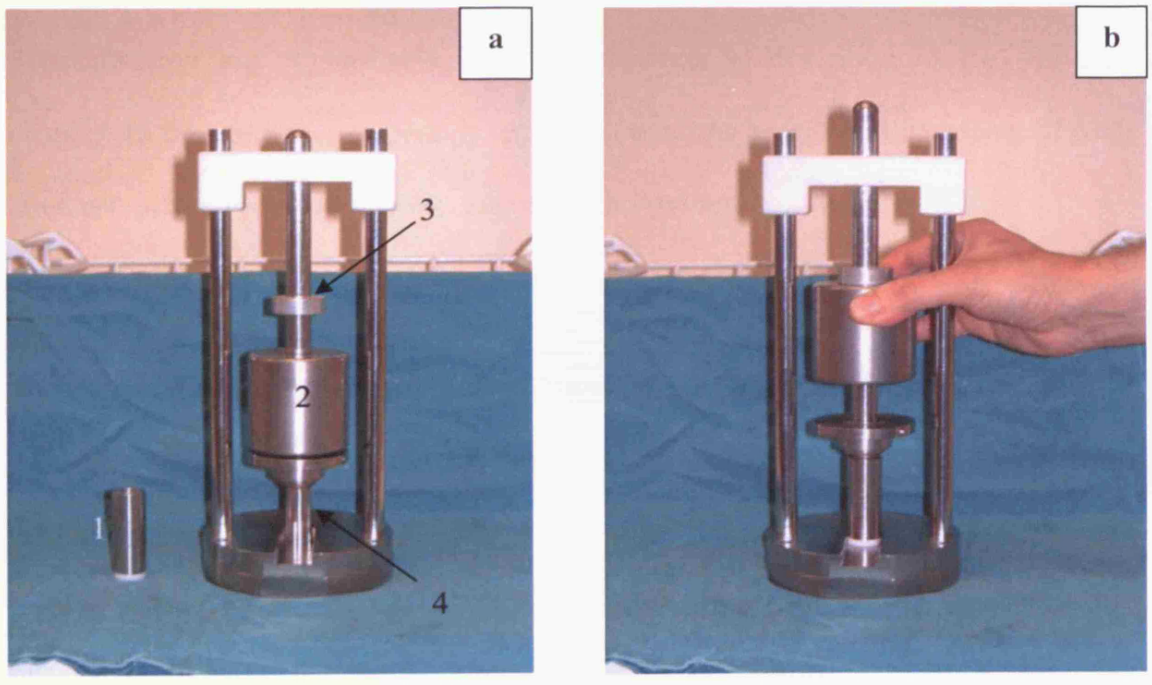


Figure 3.2: Impaction hammer design: (a) showing (1) impaction cylinder, (2) 1 kg weight, (3) stop at 2.5 cm above the weight (delivers 3kN force) and (4) site for impaction cylinder; (b) impaction cylinder placed in the hammer with impactions performed as shown

Twelve adult skeletally mature ewes (mean body weight 70.9 ± 6.5 kg) were used in the study. Each animal underwent surgery to both its medial femoral condyles, thus twenty-four operative procedures were performed. In six animals, the treatment mixture comprised of allograft + MSCs on one femoral condyle and allograft alone on the contralateral femoral condyle. In the other six animals, the treatment mixture comprised of HA + MSCs on one femoral condyle and HA alone on the contralateral femoral condyle. In this way, each subject acted as its own control.

3.2f Surgical procedure

All surgery was conducted in compliance with United Kingdom Home Office regulations. Animals were individually housed in pens, forty-eight hours prior to the surgery. Only fluid intake was permitted twelve hours before the operations. Premedication and anaesthesia was administered as described in the previous chapter. In the anaesthetic room, a large area over the antero-medial aspect of each knee was shaved and thoroughly cleaned with Povidine solution.

The sheep were placed in the lateral position on the operating table. The medial femoral condyle was approached via a 4cm incision made just proximal to the knee joint and continued along the femoral shaft. The incision was extended deeply through the subdermal fascia and muscle until bone was reached. The soft tissues were retracted using self-retainers and haemostasis established. A femoral guide plate secured with two 2.7mm cortical screws was used to guide the insertion of Tufnol ™ tubes containing the impacted graft mixtures into the defects (Fig 3.3).

Unicortical metaphyseal defects of 15mm in diameter were created, with the aid of a drill centraliser, in the distal femur at the level of the medial condyle (Fig 3.3). At each site, a 5mm diameter pilot hole was drilled in the near cortex. The depth of the defect was drilled to 4mm deeper (Fig 3.4) than the height of the pellet created by impacting the morsellised graft material (Fig 3.4). A 15mm drill was used to enlarge the unicortical defect. The Tufnol ™ tube containing the graft plug was mounted onto the guide plate and the plug gently introduced into the bone defect using a polyethylene punch (Fig 3.4). The plate and screws were removed and the soft tissues and skin closed in two layers with an absorbable suture.

The sheep were turned, re-draped and the operation repeated on the contralateral femoral condyle. Following surgery, animals were allowed immediate postoperative mobilisation and weight-bearing as tolerated. Antibiotic and analgesic prophylaxis was administered daily with subcutaneous injections of Baytril (Enrofloxacin 5mg/kg; Bayer AG Leverkusen) and Finadyne (Flunixin Meglumine 2mg/45kd; Schering-Plough Ltd) for 3 days post-surgery. All sheep were euthanased 6 weeks after surgery with an overdose of intravenous Pentobarbitone. The femur was harvested through the old incision.

3.2g Defect densitometry

After removing skin and soft tissue, the limbs were fixed in 10% formaldehyde solution and analysed with computed tomography (model XCT 2000, Stratec Medizintechnik, Germany) (Fig 3.5). Transverse scans were taken using 0.2mm-thick contiguous slices from the middle of the graft. The pictures produced by the scanner provided bone mineral density (BMD) data in the form of colour transverse

section images (Fig 3.6). Blue and black represents a BMD above 600 mg/cm^3 whilst red and grey represents a BMD below 250 mg/cm^3. Three areas on each scan were evaluated: (1) anterior host-graft interface; (2) posterior host-graft interface; and (3) middle of the graft (Fig 3.6). BMD for each of the regions was calculated separately. Data from the anterior and posterior interfaces were averaged to give one set of data for the Graft-Host Interface.

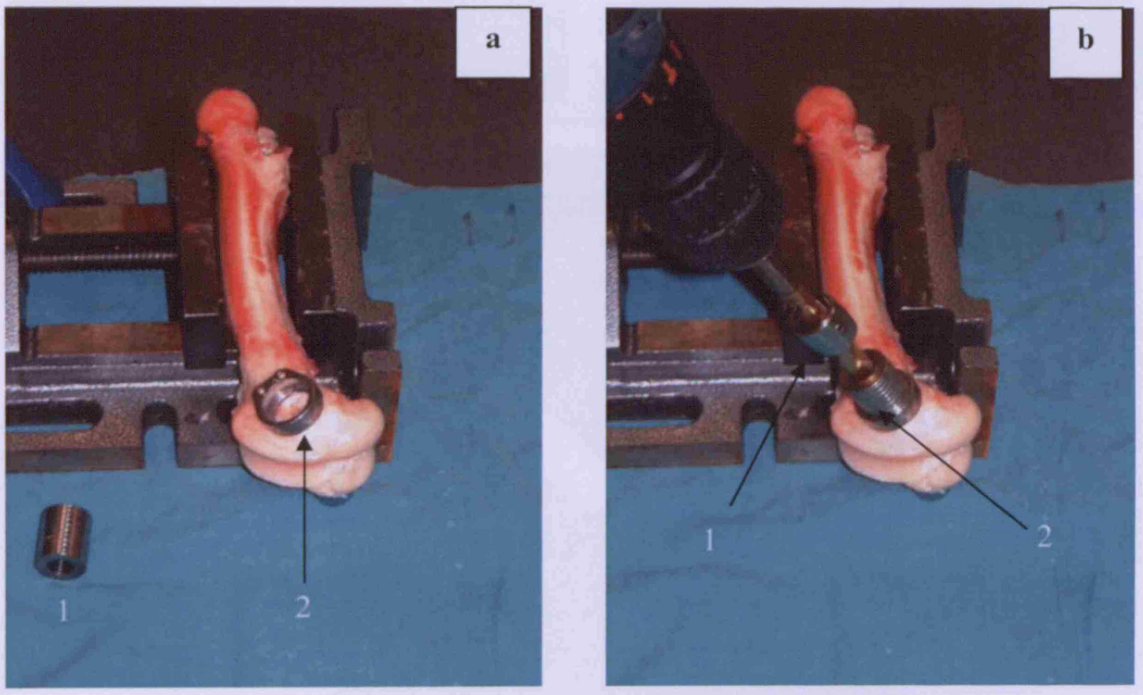


Figure 3.3: Formation of a medial femoral condyle defect: (a) a drill centraliser (1) is placed over a femoral guide plate (2); (b) a stopper (1) is used to ensure drilling to a pre-determined height.

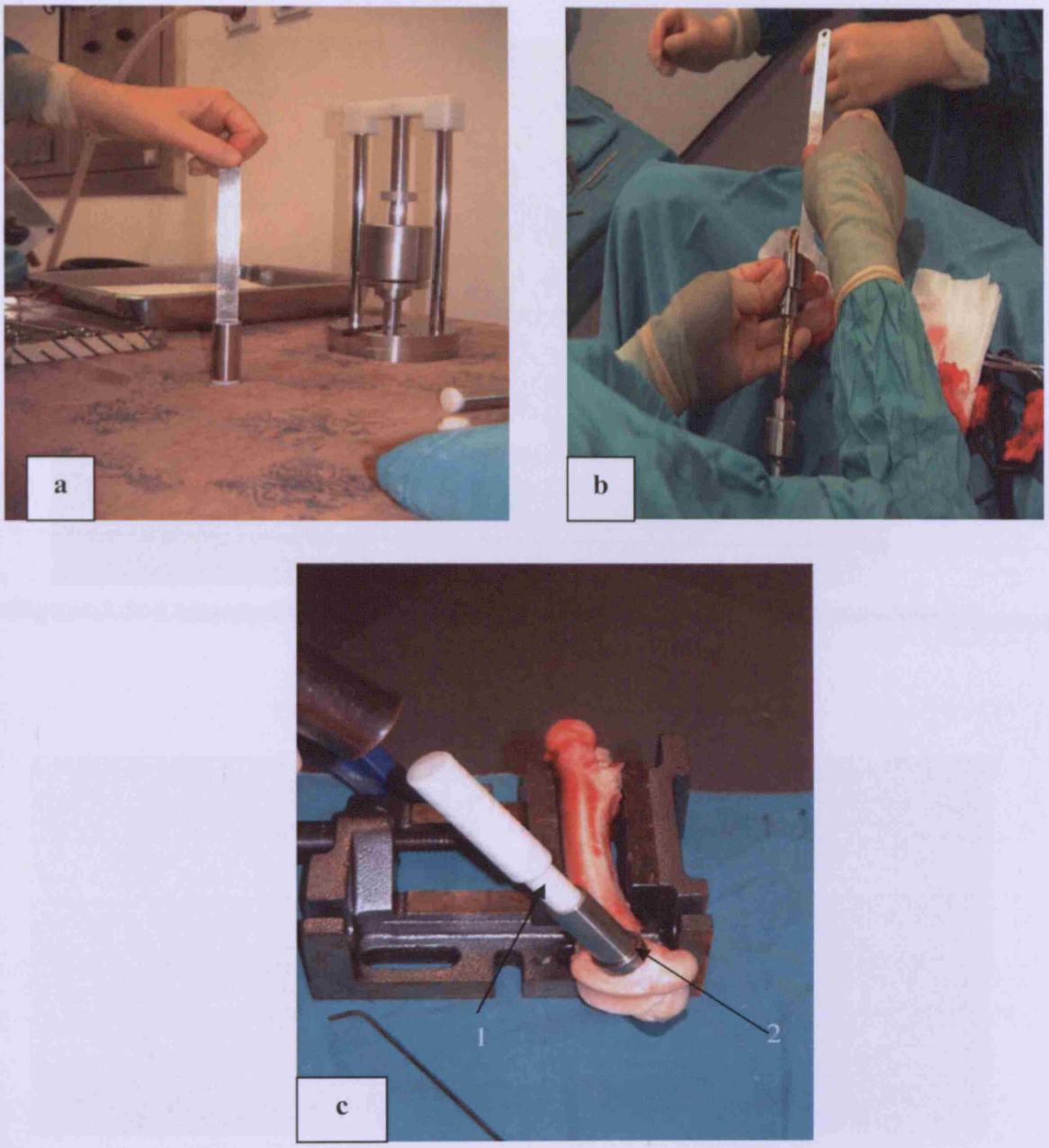


Figure 3.4: Calculation of depth of femoral condyle defect to be created. The depth of the defect was drilled to 4mm deeper (b) than the height of the pellet created by impacting the morsellised graft material (a). The Tufnol™ tube containing the graft plug was introduced into the bone defect (c) using a polyethylene punch (1).

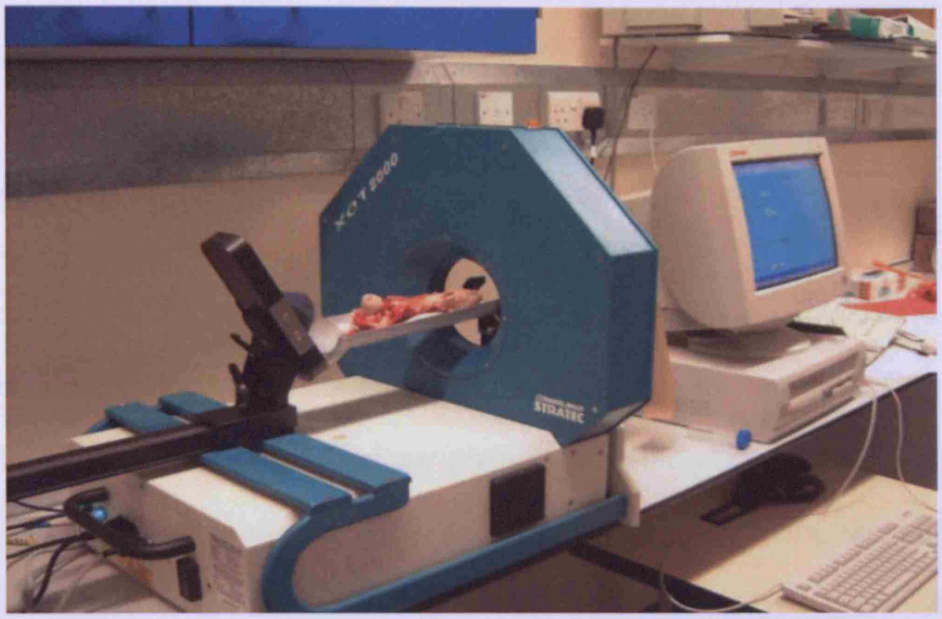


Figure 3.5: Computed tomography machine used to measure defect densitometry.

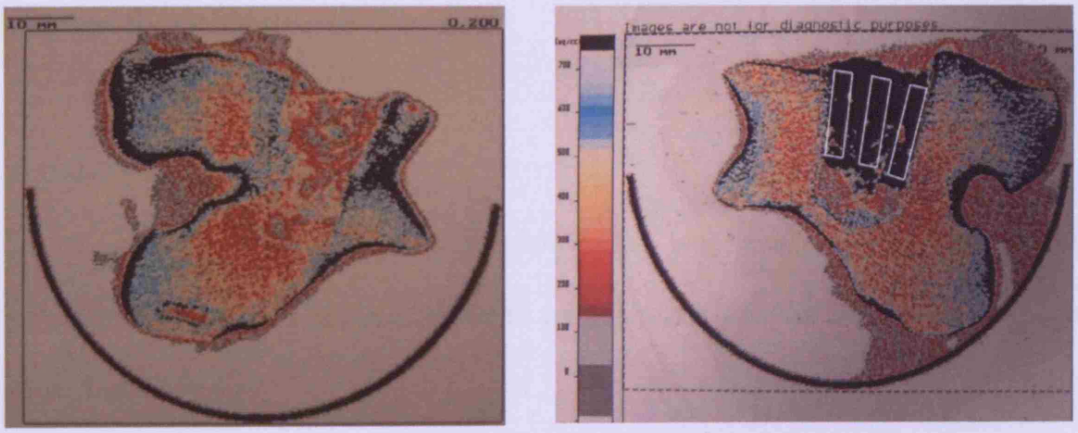


Figure 3.6: CT Images of Allograft (left) and HA (right). BMD was calculated for the 3mm wide areas marked on the right image.

3.2h Histology

Muscle and soft tissues were removed from the limbs following CT. Each bone was sectioned with a band saw to leave an area comprising of the femoral condyles.

Initially the samples were fixed in formal saline[1] and then prepared for histology in a similar fashion to that described in Chapter 2.

3.2i Histomorphometry

The slides were examined in three regions in the same manner as the BMD estimations: (1) anterior host-graft interface; (2) posterior host-graft interface; and (3) middle of the graft (Fig 3.7). Four pictures were taken at random in each region of analysis (twelve pictures per slide), the area fraction of original graft (allograft or HA) and new bone were measured using the KS 300 image analysis system (Fig 3.7). The data is represented as percentage of remaining graft and percentage of new bone formation. The data for the anterior and posterior interfaces were averaged to give one set of data for the Graft-Host Interface.

3.2j Statistics

Bone mineral density and histomorphometric data were analysed using the SPSS (v 10.1) statistics software and subjected to paired t-test. Results are shown as mean ± standard mean error.

[1] BDH Laboratory supplies

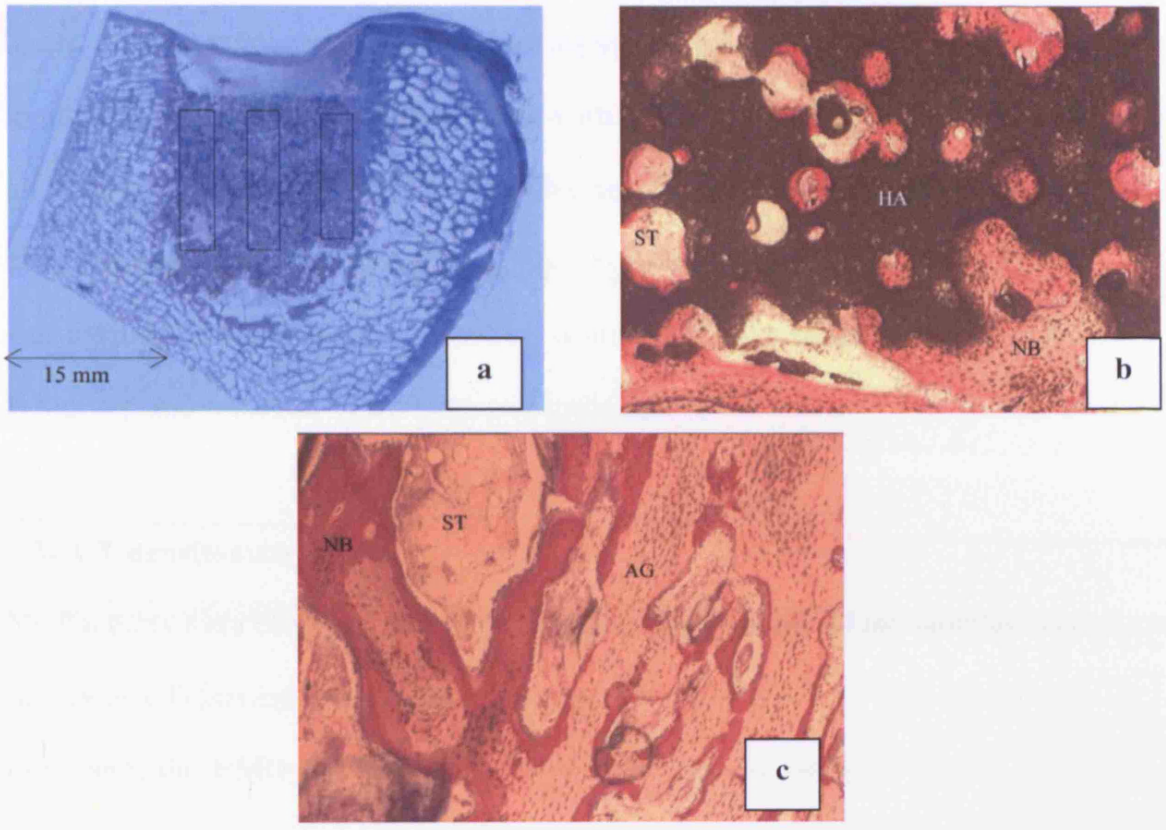


Figure 3.7: Histological analysis of specimens: (a) sections stained with paragon and toluidine blue with areas to be analysed demarcated by the three rectangles; (b) x20 magnification histology sections of HA granules (HA) and (c) allograft (AG) showing new bone (NB) formation and soft tissue (ST).

3.3 Results

There were no postoperative complications and no evidence of implant rejection in any of the animals. One defect filled with HA (control group) was devoid of all treatment material and did not exhibit any evidence of graft incorporation. This was most likely because the graft had become dislodged and this specimen was excluded from the study. Therefore, results were analysed on 5 defects in the HA group and 6 defects in the stem cell seeded group.

3.3a CT densitometry

No fractures were observed on computed tomography in any of the samples. The results of CT-derived densitometry are summarised in Table 3.1 and Fig 3.8. At time zero, the BMD for HA was 1150 mg/cm^3 and for allograft it was 297.6 mg/cm^3. There was no significant difference in new bone formation between the host-implant interface and the middle of the graft in either the HA or allograft groups. Similarly, no significant new bone formation was observed between the stem cell seeded graft and the control side at either region.

3.3b Histomorphometry

The porosity of the un-impacted graft was 60% for HA and 61% for allograft which, after 20 impactions with 3kN, reduced to 51% ($p>0.05$) and 5.7% ($p<0.01$) respectively. At time zero, 49% of the defect was filled with HA and 39% with allograft. After six weeks, this remained unchanged for HA but only 5.7% ($p<0.01$) of the defect was filled with allograft signifying that most of it had been resorbed (Fig. 3.9). The results of histomorphometry are summarised in Table 3.2 and Fig 3.10. In both the HA and allograft groups (test and control),

there was no significant difference in new bone formation in either the middle of the graft or at the host-implant interface (Fig 3.10). In all the groups, new bone formation was more prominent at the host-graft interface compared with the middle of the graft (Figs 3.11 & 3.12). With the HA group, new bone formed in between the HA granules as well as in some of the pores within the granules (Fig 3.11). In the middle of the implant, less new bone formation was observed and presence of fibrous tissue was evident (Fig. 3.11).

Treatment	Location	Test/Control group	BMD *(mg/cm^3)*	P value
HA	Middle of graft	Test	1114 ± 25.1	> 0.05
		Control	1122 ± 51.2	
	Graft-host interface	Test	1069 ± 39.0	> 0.05
		Control	1123 ± 39.1	
Allograft	Middle of graft	Test	243.2 ± 24.9	> 0.05
		Control	259.0 ± 16.7	
	Graft-host interface	Test	304.1 ± 41.2	> 0.05
		Control	255.3 ± 22.3	

Table 3.1: Bone Mineral Density (BMD) values for the HA and allograft groups.

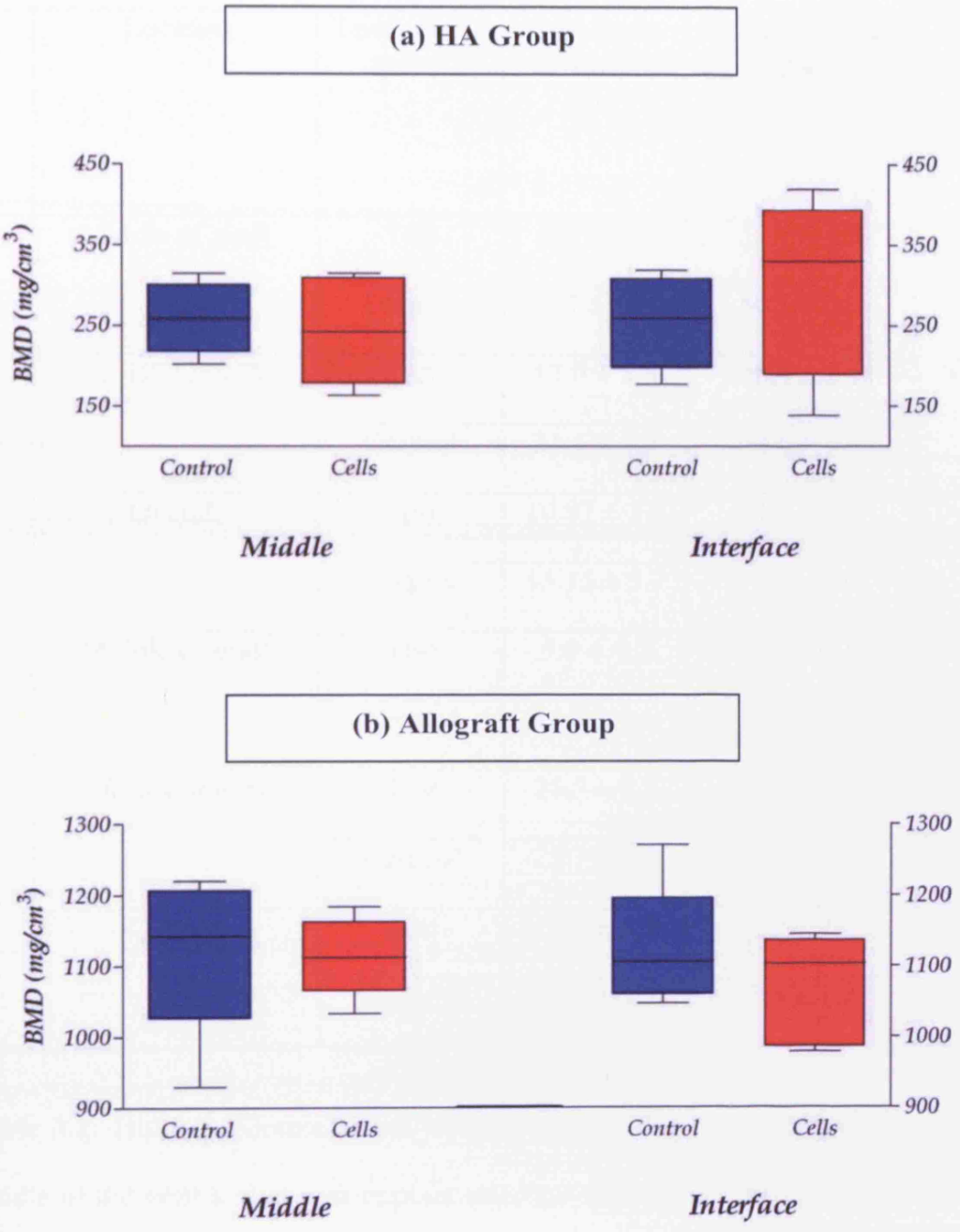


Figure 3.8: Bone Mineral Density for (a) HA and (b) allograft groups. The distance between the lowest and highest horizontal lines and the edge of the coloured box is the bottom and top quartile range respectively; the box shows the interquartile range. The thick black line in the middle of the box represents the median.

Treatment	Location	Test/Control group	New Bone Formation (%)	Remaining graft (%)	P value
HA	Middle of graft	Test	5.0 ± 2.6	51.7 ± 1.9	> 0.05
		Control	7.3 ± 4.1	50.1 ± 2.0	
	Graft-host interface	Test	17.0 ± 5.8	50.4 ± 2.4	> 0.05
		Control	21.1 ± 4.5	48.6 ± 1.7	
	Overall	Test	10.87 ± 3.6	51.1 ± 0.7	> 0.05
		Control	15.15 ± 3.7	49.35 ± 0.8	
Allograft	Middle of graft	Test	9.9 ± 4.2	9.5 ± 4.1	> 0.05
		Control	11.0 ± 2.1	5.7 ± 2.0	
	Graft-host interface	Test	25.5 ± 8.2	6.4 ± 2.1	> 0.05
		Control	23.7 ± 5.4	8.9 ± 1.6	
	Overall	Test	16.8 ± 3.9	7.9 ± 2.3	> 0.05
		Control	18.8 ± 2.7	7.3 ± 1.3	

Table 3.2: Histomorphometric analysis: percentage new bone formation in the middle of the graft and at host-implant interface for defects filled with impacted HA and allograft (Control Group) and impacted stem cells seeded on HA or allograft scaffold (Test Group).

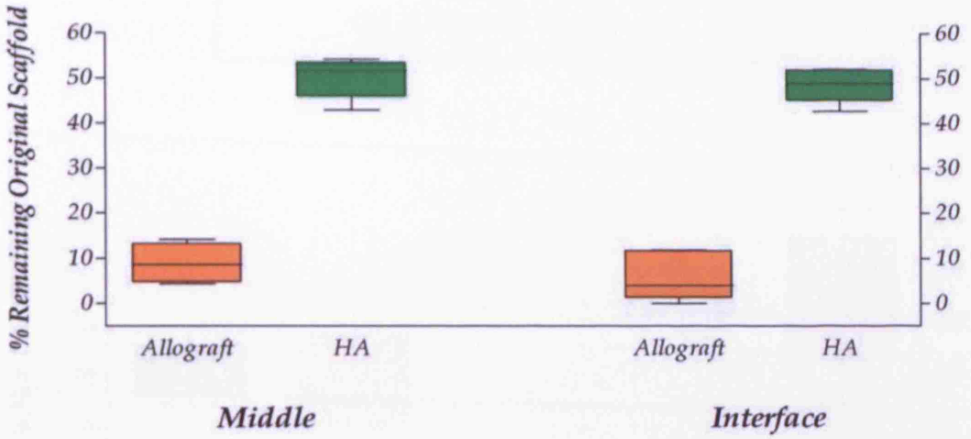
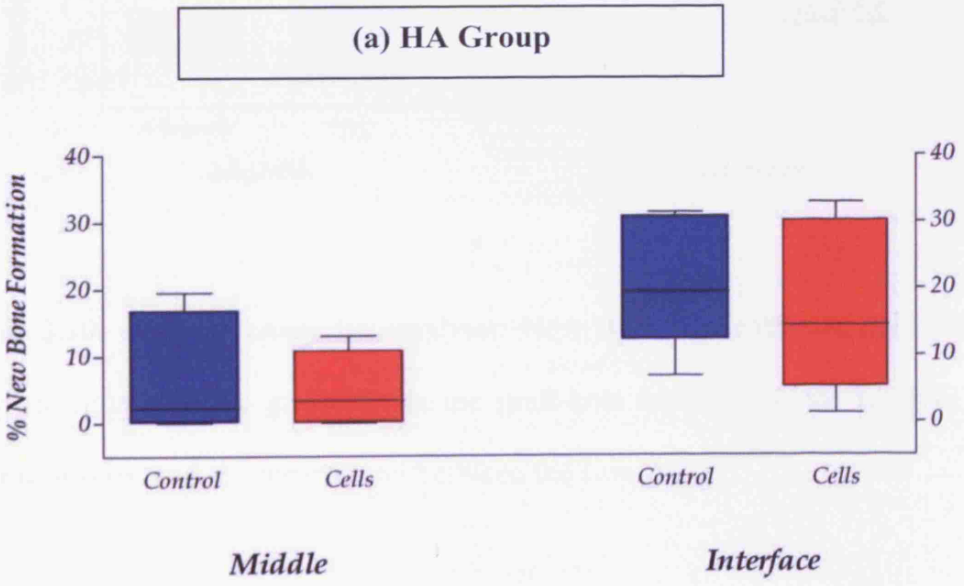


Figure 3.9: Amount of remaining scaffold at six weeks (values expressed as percentage).



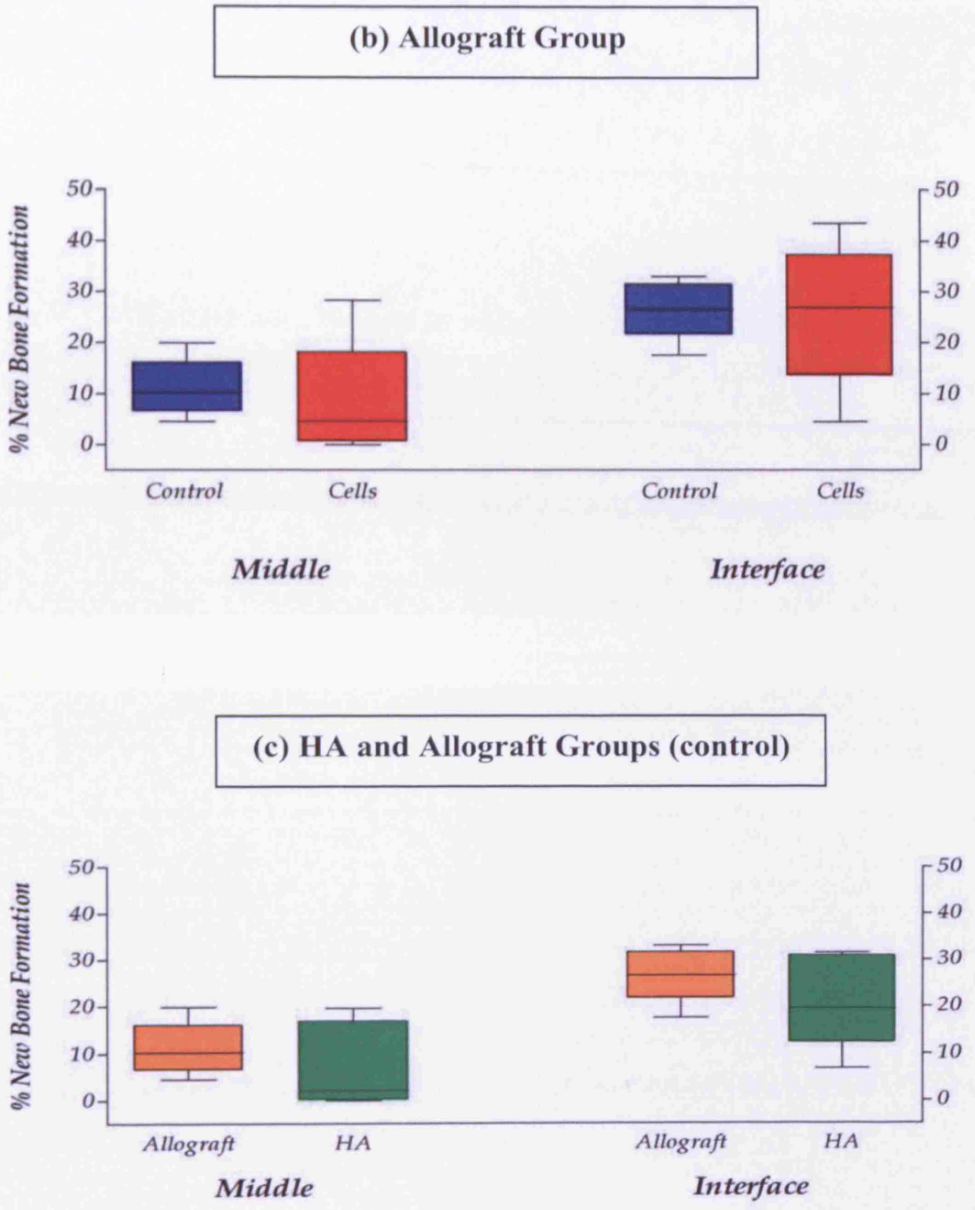


Figure 3.10: Histomorphometric analysis: New Bone Formation in the control group in middle of the graft and at the graft-host interface in the (a) HA (b) allograft groups and (c) comparison between the two groups.

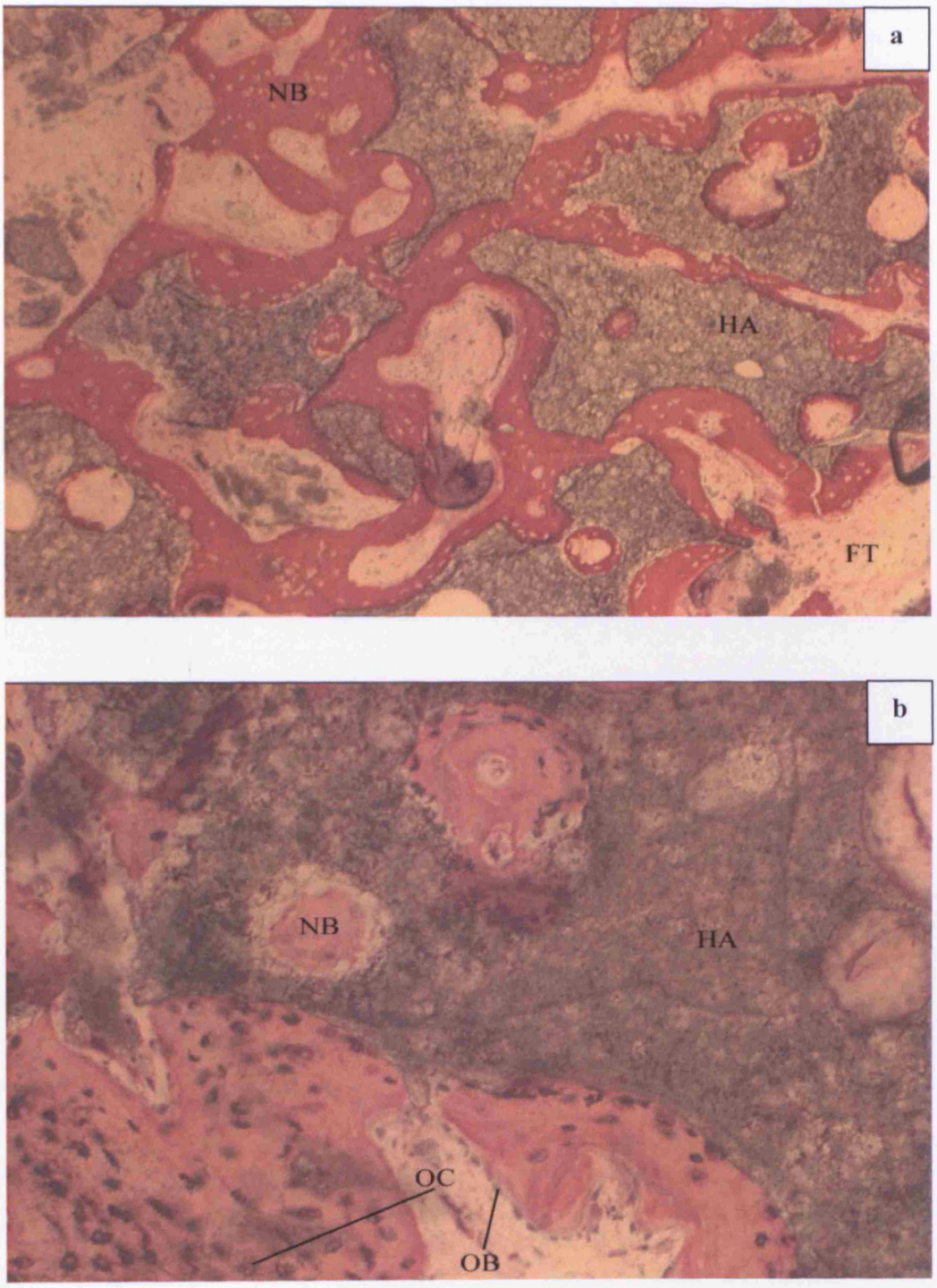


Figure 3.11: Histological analysis within the HA group (a) x20 magnification (b) x50 magnification New bone (NB) bridging the gaps between the HA granules. Osteoblasts (OB) line the surface of newly formed bone. Osteocytes (OC) are trapped within the mineralised osteoid. Near the middle of the graft, bone formation was present but instead was replaced by fibrous tissue (FT).

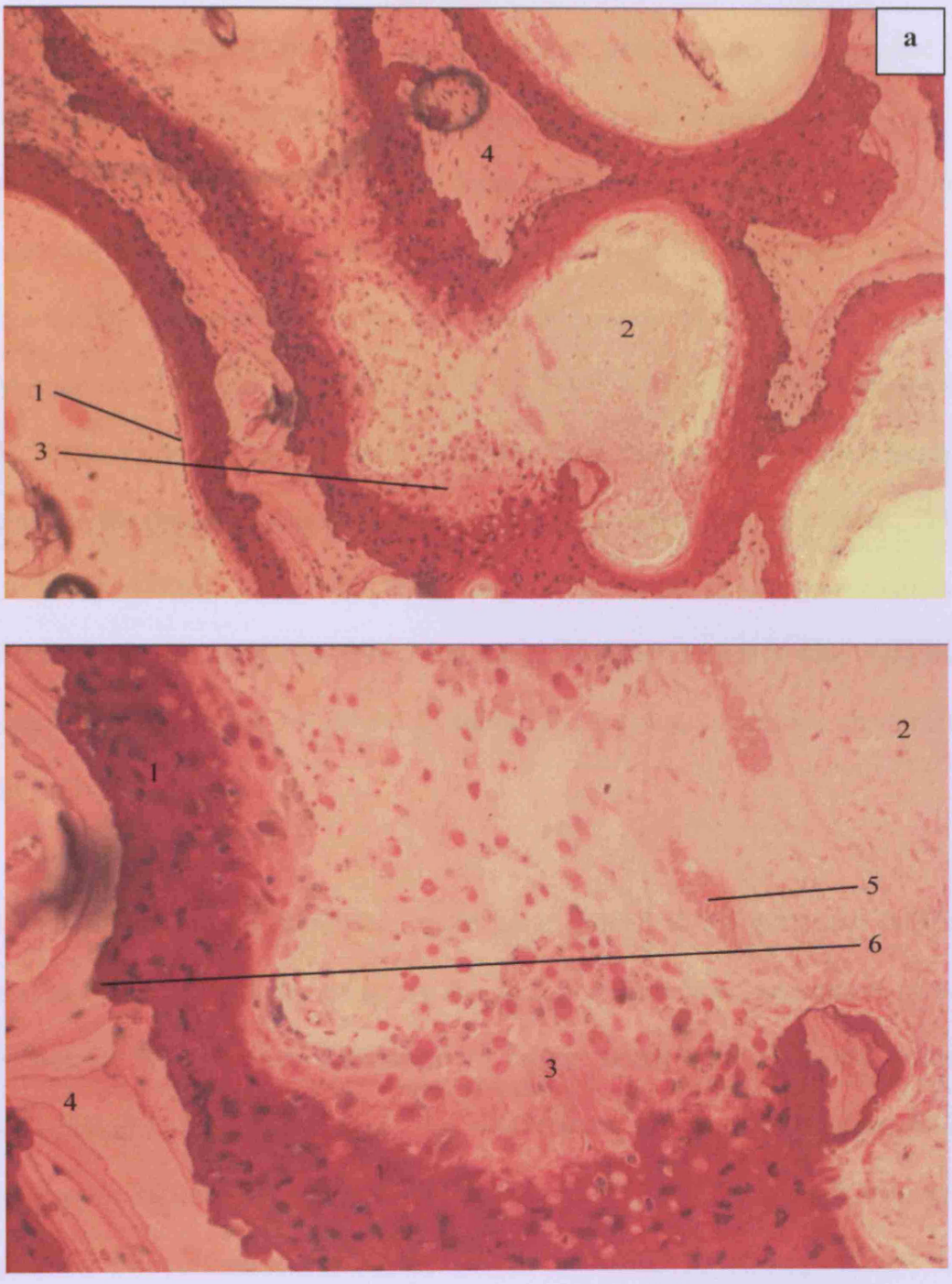


Figure 3.12: New bone formation in impacted allograft group (a) x20 magnification and (b) x50 magnification. (1) Osteoblasts covering the surface of newly formed bone (2) Primitive marrow cavity (3) Intramembranous ossification (4) Old allograft (5) newly formed blood vessels and (6) Howship's lacunae.

3.4 Discussion

An ovine metaphyseal femoral unicortical bone defect model was selected for this study. This is a suitable model for several reasons: it maximises the use of animals; does not require mechanical fixation; allows evaluation of large particle materials; and sheep stem cells have been shown to be a valuable model in determining the biocompatibility and osteointegration of orthopaedic materials (Morgan and Leopold, 2004; Griffon et al., 2001; Torricelli et al., 2000; Kon et al., 2000). Furthermore, the distal femur has been shown to be an ideal location to assess the incorporation of biomaterials and osteogenesis (Griffon et al., 2001; Lu et al., 1998). However, while simulating some of the environmental characteristics encountered in revision hip arthroplasty (contact with cancellous bone), this model did not intend to mimic the loading conditions encountered clinically.

Within all the groups, there was more bone formed at the graft-host interface than in the middle of the graft. At the graft-host interface, recruitment of host osteoblasts is an expected response to injury. If the stem cells had made a contribution to bone formation, the single most important difference in the pattern of new bone formation would have been filling of the internal macropore space with bone formed directly by introduced MSCs which had differentiated into osteoblasts. However, other studies (Kon et al., 2000) evaluating the effects of autologous bone marrow stromal cells loaded onto biomaterials have also shown a similar pattern of bone growth. Possible explanations for this include (a) an increased density of loaded cells within the peripheral regions of the composite graft (b) superior survival of the loaded cells at the graft-host

interface, which are possibly vascularised earlier and more effectively than the middle regions and (c) stimulation of endogenous osteoprogenitor cells, within the host tissues, by the implanted cells via a positive feedback mechanism.

The lack of contribution to bone formation by stem cells, in this study, contrasts with results observed by others (Kon et al., 2000) who showed that cells loaded onto porous HA scaffold produced a far more extensive bone formation than cell free implants in tibial defects in sheep at 8 weeks. However, there are several noticeable differences between their study and this one. Firstly, Kon et al used an external fixator to achieve mechanical stability and this could potentially cause mechanical stimulation of both implants and bone resected ends. Secondly, they used fibrin as a cell sealant, which itself has been shown to promote osteogenesis from the host osteoprogenitor cells (Fortunato et al., 1997). Finally, their scaffold consisted of HA cylinders and not granules, as used in this study. The morphology of the scaffold has been shown to be crucial in tissue engineering and is discussed in the next chapter.

Another important difference between my study and that of Kon et al is that I impacted cells whereas they used un-impacted cells. Intra-operative measurement of forces during femoral impaction allografting in revision hip surgery have shown a range of 3 to 9 kN (Phipps et al., 2002) with the majority of the impactions being between 3 to 6 kN. However, there is a wide variability in impaction forces generated by different surgeons and also by the same surgeon operating on different patients (Phipps et al., 2002). Therefore, a 3kN impaction force used in this study may not be representative of the average impaction force generated during revision hip surgery. Although, stem cells are able to survive

the impaction process in-vitro (Korda et al., 2006), the purpose of this study was to evaluate if a similar finding would be observed in-vivo and therefore a smaller impaction force of 3kN was used.

Another important factor contributing to the lack of new bone formation in this study may be the osteogenic potential of the site of implantation. In this study, the samples were impacted into a site where the blood supply was good and where there was an abundance of osteoblasts and probably other stem cells from the host bone to support bone formation. So it may be that in both situations bone formation was at an optimal level and other factors prevented the stems cells from having a significant effect. It maybe that in a site where osteoblasts are reduced in number, the effect of stem cells would be more pronounced. However, this theory warrants further investigation.

One study that has shown success using stem cells loaded onto a HA/TCP scaffold was in a goat model (Kruyt et al., 2003). However, in this study, the investigators were unable to prove that the implanted cells were present in the bone that subsequently formed, due to the absence of a reliable tracing method to mark their cells. It is also plausible that their osteoinductive scaffold could have stimulated osteogenic differentiation of cells. Although the mechanism responsible for this theory remains a speculation, it is well established that osteogenesis occurs in well-defined cavities (Ripamonti, 1996). It is therefore possible that in such cavities, there occurs an accumulation of factors that are fundamental for induction and/or differentiation of the cells (Yamasaki and Sakai, 1992). In addition, such a microenvironment may support calcium phosphate break down and reprecipitation, possibly in combination with crucial

factors. Such an induction phenomenon can be supported by the finding that osteogenesis inside arteriosclerotic plaques only occurs as a final stage, inside calcified (HA) plaques (Deneke et al., 2001).

The method used for the isolation and culture of human MSCs in our laboratories has yielded cells that have successfully been differentiated into adipocytes, chondrocytes and osteoblasts (Kalia et al., 2006). Furthermore, cells loaded onto an allograft scaffold have been shown to be capable of differentiating into osteoblasts (Rust et al., 2007). My seeding technique was validated by scanning electron microscopy, showing the attachment of the seeded cells on to allograft and HA granules. However, the seeding efficiency was not verified. Thus, although $2x10^6$ cells were seeded onto the scaffold, there could have been a reduction in the number of cells on the graft at the time of implantation. Furthermore, 2×10^6 cells per cm^3 of scaffold is a much lower density than that used by others who have demonstrated success with the use of stem cells (Kruyt et al., 2003; Bruder et al., 1998). It is possible therefore that we had an insufficient quantity of stem cells in the composite grafts to enhanced bone formation.

Once seeded with cells, the graft was incubated for 4 days prior to implantation to allow the cells to lay down extracellular matrix. Recent evidence suggests that viable cells rather than the extracellular matrix are essential for bone formation (Kruyt et al., 2003) and therefore, many authors favour seeding the graft at the time of implantation (Bruder et al., 1998). However, results from other studies (Mendes et al., 2002) have shown that scaffolds on which cells have been seeded

for up to 5 days before implantation, induce faster bone formation compared with scaffolds in which cells are seeded and implanted a few hours later.

Most studies using mesenchymal stem cells have used alkaline phosphatase (ALP), which is an enzyme tightly bound to the cell membrane, as a marker of osteoinductive cells. A peak of ALP activity is found to precede the increase in the rate of mineralisation. However, as in other studies (Kon et al., 2000), we found that ALP activity was almost undetectable in sheep MSCs and at the time of the study there was no method to confirm the presence of osteoinductive cells. Furthermore, at the time of this study there was no reliable tracing method to mark the MSCs and this posed a major difficulty in determining if stem cells had contributed to new bone formation. Currently, studies are on going at our Institute to examine the use of Green Fluorescent Protein (GFP) and lacZ as potential markers to trace stem cells.

Inoue et al. (1997) studied the effect of aging on osteoblastic differentiation of marrow stromal cells loaded onto porous hydroxyapatite in rats. They found reduced bone formation in older bone marrow cells and concluded that this may have been due to a lesser stromal cell population and /or the reduced potential of stromal cells to differentiate down the osteogenic pathway. Nishimoto et al. (1985) assessed ectopic bone formation by subcutaneously implanting crude BMP powder in rats of different ages. High ALP activity appeared later in older rats compared with younger rats, and ALP activity was decreased in older rats. In this study, sheep with a variable age range were used (average age 2-4 years) and it is possible that this could have adversely affected the stem cell population and /or their differentiation capability, resulting in the results observed.

Following implantation, the graft is incorporated in a process that is similar to that of fracture healing (Tagil, 2000) which is initiated by haematoma formation at the graft site. Incubation of endothelial cells in fracture haematoma supernatant has been shown to significantly inhibit the in vitro angiogenic parameters of endothelial cell proliferation and microtubule formation (Street et al., 2000). Furthermore, fracture haematoma potassium concentration is known to be cytotoxic to endothelial cells and osteoblasts (Street et al., 2000). It is therefore possible that the local environment within the defect proved too harsh for the stem cells to survive in.

Although most of the clinical series have evaluated the cemented impaction allografting technique (Mikhail et al., 1999; Meding et al., 1997; Elting et al., 1995; Leopold et al., 1999; Gie et al., 1993), this study did not use cement because of the potential risks of destroying the stem cells from the heat generated by the cement. Defects were only covered with the vastus medialis muscle. This permitted the ingrowth of fibrous tissue especially in the allograft group. Presence of fibrous tissue has been shown to delay or impede new bone formation (Gosain et al., 2004) and could also have destroyed the stem cells. Tissue Guided Regeneration (TGR) is a technique that has been used to overcome this particular problem by using barrier membranes such as polytetrafluoroethylene (PTFE), to prevent the prolapse of adjacent tissues into the defects (Gosain et al., 2004).

In this study, the animals were euthanased at 6 weeks. The majority of investigators using TE constructs have evaluated their specimens after a longer

period (Kruyt et al., 2003; Yoshikawa et al., 1998; Boo et al., 2002; Kadiyala et al., 1997; Kon et al., 2000). It can only be speculated whether more bone formation would have been observed if this study was conducted over a longer period. However, other studies have shown more bone formation in the TE constructs as early as 4 weeks after implantation of their grafts (Bruder et al., 1998).

The impaction forces used resulted in the graft comprising of HA to be more compacted than the allograft. In the control groups, there was no statistical difference in the amount of new bone formed by the granular porous HA or allograft. In both these groups, more new bone formation was detected at the host-implant interface than the middle of the implant at six weeks ($p < 0.05$). These results are similar to those observed by Heekin et al. (1995) and Won et al (2004) who analysed autopsy retrievals of acetabular reconstructions with allograft and HA respectively. In both these studies, bone penetration only occurred at the peripheral junction of the graft with cortical bone and the central graft had only myxofibrous tissue with no live bone, as long as 18 months postoperatively. The current report demonstrates that hydroxyapatite does not need to be mixed with allograft to induce adequate bone formation. However, the tendency for bone formation to occur only in restricted regions remains a concern because this could hinder continuous bone formation that is, for example, mandatory in spinal fusion. Furthermore, central defects that only provide soft tissue contact for morsellised allograft or HA could compromise the stability of the graft.

There was a significant difference in the rate of resorption between the allograft and HA groups at six weeks, with the allograft being almost completely resorbed whilst there was minimal resorption of HA. An ideal bone graft substitute material from both a biological and biomechanical point of view is one that is completely replaced by new bone formation from the host. However, in order for the graft to maintain or increase structural properties, the graft material has to be replaced by bone formation. If resorption is faster than bone formation, then the structural integrity of the graft may be compromised. This study confirms previous reports (Hyakuna et al., 1990) that although HA is an excellent bone substitute, it does not replace new bone because of its biodegradable characteristics as a scaffold. In my study I showed that degradation of the HA was much slower than the allograft. Moreover, the HA became bound to the host bone (Jarcho et al., 1977) and remained connected to it via new bone that formed into the interconnected interpores (Won et al., 2004; Bolder et al., 2003). Resorption of allograft in this model allowed greater ingrowth of fibrous tissue. This could potentially compromise the stability of the construct. The HA was not resorbed after 6 weeks and hence may be more stable.

3.5 Conclusion

Impacted hydroxyapatite (HA) induces the same amount of bone growth as impacted allograft. Although mesenchymal stem cells (MSCs) have been shown to survive the standard impaction forces measured at revision hip surgery in an in-vitro model, this study showed that the addition of MSCs to allograft or HA granules did not enhance more bone formation in-vivo. This may be because stem cells lose their differentiating capacity after impaction. This warrants further investigation and forms the basis of the next chapter.

CHAPTER 4

THE EFFECT OF MESENCHYMAL STEM CELLS ON NEW BONE FORMATION IN IMPACTION GRAFTING: AN IN VIVO ECTOPIC MODEL

4.1 Introduction

To date, many studies have been performed to optimise the isolation, expansion and seeding of mesenchymal stem cells to increase their bone-forming capacity (Bruder et al., 1998; Gundle et al., 1995; Dennis et al., 1992). It has been shown that combining MSCs with a porous ceramic scaffold is a feasible concept to generate bone ectopically, or in critical sized defects in rodents (Ohgushi et al., 1989; Yoshikawa et al., 2000; Bruder et al., 1998; Bruder et al., 1997). However, transferring this technology to larger animals has been challenging (Decker et al., 1979; Deleu and Trueta, 1965) and only a few reports have demonstrated bone formation orthotopically (Bruder et al., 1998; Kon et al., 2000) or ectopically in rabbits, dogs and goat (Anselme et al., 1999; Peter et al., 2000; Kruyt et al., 2003).

One reason that bone tissue engineering has appeared to be much more difficult in larger animals might be due to impaired cell survival, as the metabolic rate of these animals is lower and/or larger implants are required. In addition, experimental defects created in larger animals are usually bigger and this has consequences in terms of vascular invasion and survival of cells deep within the larger scaffolds. These potential reasons are supported by previous studies that report negligible cell survival and delayed revascularisation of bone grafts after auto-transplantation (Decker et al., 1979; Deleu and Trueta, 1965; Nettelblad et al., 1984; Redondo et al., 1997). In addition, there is a lack of vascularisation until the first week after implantation, which may compromise cell survival (Decker et al., 1979; Deleu and Trueta, 1965). This could explain why there was no effect of the mesenchymal cells on bone formation in the bone defect site reported in the previous chapter.

Research at this institute has shown sheep MSCs are able to survive impaction forces up to 6 kN (Korda et al., 2006). In the previous study, a smaller impaction force of 3kN was used to ensure cell survival. However, it is possible that although stem cells survive impaction, their differentiating capacity is reduced as a result of the impaction process. This theory warrants further investigation.

In the previous study a metaphyseal femoral condyle defect model was used to investigate the osseoinductive capacity of impacted TE constructs. Although this model has several advantages, the environment does not simulate the conditions encountered clinically during a revision situation where impaction grafting would be used. For example, this area has a good blood supply and does not mimic the poor bone stock encountered in a clinical revision situation. Furthermore, within the sheep condyle the environment is very conducive to bone formation and any effect that the stem cells would have had may have been masked. To rule out osteoconduction or periosteal bone formation (Bruder et al., 1998; Kon et al., 2000) as confounding and overriding mechanisms of bone formation, ectopic implantation sites have been used successfully to investigate the bone-forming osseoinductive capacity of tissue-engineered constructs implanted in goats (Kruyt et al., 2003). To our knowledge, there has been no documented use of ectopically implanted tissue engineered scaffolds in sheep. An extra-skeletal model would indicate the osseoinductive nature of the combined scaffold and stem cells and has the added benefit of allowing the investigation of many variables in one animal.

4.1a Hypothesis

This pilot study will explore the hypothesis that the addition of MSCs to allograft or HA granules will enhance the amount of new bone formation when compared with allograft or HA granules alone in an ectopic site and that impaction grafting using graft and stem cells will not result in a reduction of bone formation.

4.1b Objectives

Ectopic bone formation will be assessed by histology and histomorphometry of non-decalcified sections to generate data within the graft for new bone formation and bone attachment to the scaffold.

4.2 Methods

4.2a Experimental design

Two skeletally mature ewes (mean body weight 70.9 ± 3.5 kg) were used in this initial study. The purification, expansion and culture of MSCs onto the graft material have been described previously. All cells were confluent at passage 3; 10×10^6 cells/g were seeded onto the scaffold. Two scaffold types, allograft and HA60/400 (HA with 60% interconnected macroporosity and an average pore size of 400μm) were investigated and divided into eight treatment units (Table 4.1). For unimpacted samples, sufficient scaffold was used to fill a volume of $3cm^3$ and then seeded with the same volume of plasma +/- MSCs as the impacted group. One unit of each treatment was transferred into a stainless steel mesh and the mesh sutured at each end. Each mesh sample was randomly allocated to one of 8 implantation positions in the paraspinal muscle (4 sites either side of the spine) of the sheep from which the MSCs were derived (autologous implantation).

4.2b Surgical procedure

All surgery was conducted in compliance with the 1986 Animal Scientific Procedures Act as regulated by the United Kingdom Home Office. Pre-operative preparation was similar to that described in the previous chapters. The surgical procedures were performed under general inhalational anaesthesia, preceded by intravenous Ketamine and/or Midazolam (Roche Products, UK) and maintained by inhalation of Halothane (Meriel Animal Health Ltd) as described previously.

	Sample composition	Sample type
Impacted group	Allograft alone	Control
	Allograft with MSCs	Test
	HA	Control
	HA with MSCs	Test
Unimpacted group	Allograft alone	Control
	Allograft with MSCs	Test
	HA	Control
	HA with MSCs	Test

Table 4.1: Composition of the eight treatment units used in this study

In the anaesthetic room, the lumbar area was shaved and thoroughly cleaned with Povidine solution. The sheep was placed in a prone position on the operating table and four skin incisions (approximately 3cm in length) were made over the body of the paraspinalis muscle (Fig 4.1). The muscle epimysium was exposed and divided. Using blunt dissection, an intramuscular pocket was created, which was filled with one of the four treatment units. The epimysium was closed with 2/0 non-absorbable ethylon suture to facilitate implant localisation at explantation. The skin was closed in 2 layers with an absorbable vicryl suture. Post-operative care was similar to that described previously and included prophylactic antibiotics and analgesia. There were no post-operative complications and no evidence of implant rejection. By the second postoperative day, the sheep were moving around normally. Both sheep were euthanased 12 weeks after surgery with an overdose of intravenous Pentobarbitone.

Figure 4.1: Ectopic implantation of graft in sheep. Four incisions were made to access both the paraspinalis muscle groups.

4.2c Histology and histomorphometry

At explantation, the units were localised, the surrounding muscle tissue excised, and the constructs placed in10% buffered formal saline. The samples were prepared for histology as described in the previous chapters. The sections were examined using a fluorescence/light microscope with a double filter block. Digital photographs of the whole sample were taken using a low power x40 magnification. Area of new bone formation and remaining scaffold (allograft or HA) were quantified using the Image Tool 3.0 software (UTHSCSA, Texas, USA). The percentage of new bone formation was expressed as the amount of new bone divided by the total area available for new bone generation within the area enclosed by the mesh. Statistical analysis was not performed due to small sample number.

4.3 Results

4.3a Allograft group

In both the impacted and unimpacted groups, there was more new bone formation when allograft was seeded with mesenchymal stem cells compared with allograft alone (Fig 4. 2, Table 4.2). In the impacted samples there appeared to be more bone formation than in the unimpacted samples. New bone formed on the surface and in the pores of partially degraded allograft. In some cases, new bone bridged between separate segments of allograft. There were also areas of unmineralised osteoid and ongoing ossification, not yet matured into new bone (Figs 4.3 & 4.4).

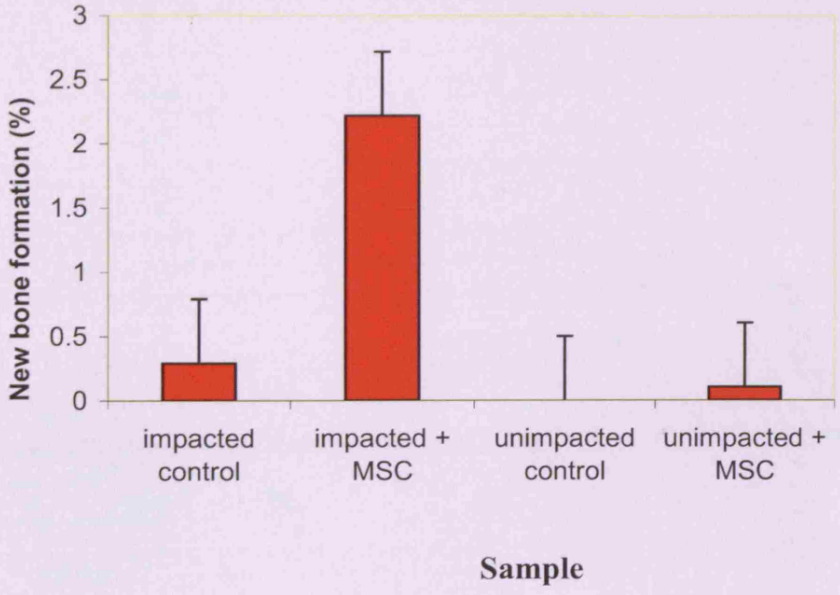


Figure 4.2: Graph showing percentage new bone in different allograft groups.

	New bone formation (%)		
Sample	1	2	Mean of two samples
Impacted allograft (control)	0	0.58	0.29
Impacted allograft with MSC's (test)	1.22	3.22	2.22
Unimpacted allograft (control)	0	0	0
Unimpacted allograft with MSC's (test)	0	0.21	0.105

Table 4.2: Percentage new bone formation in impacted and unimpacted allograft samples

Figure 4.3: New bone (a) forming on allograft (c) with areas of ongoing ossification (b) (x 40 magnification).

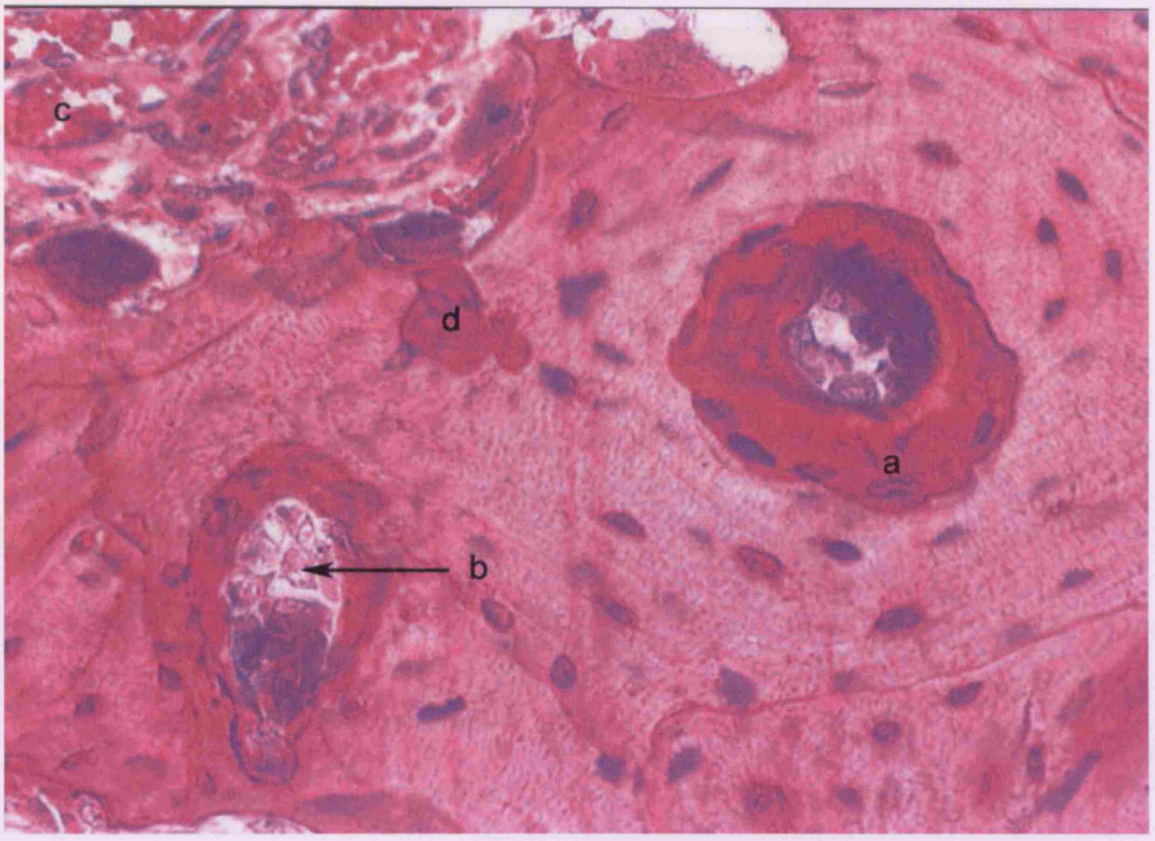


Figure 4.4: High magnification image (x100) of new bone formation within pores of allograft (a). Osteoblasts (b) line the surface of newly formed bone. Angiogenesis (c) is evident as is new bone in areas excavated by osteoclasts (d).

4.3b Hydroxyapatite group

There was no new bone formation in either the impacted or unimpacted hydroxyapatite groups. Although there was evidence of angiogenesis, pores were filled with fibrous tissue, with no evidence of any unmineralised osteoid (Fig 4.5).

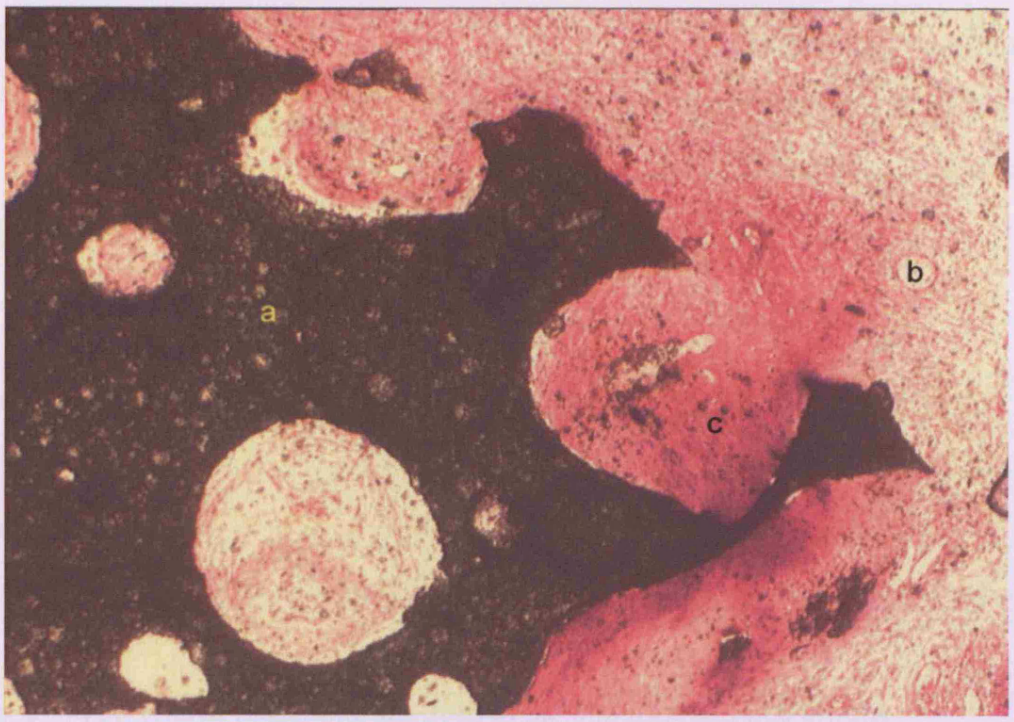


Figure 4.5: Impacted hydroxyapatite (a) demonstrating evidence of angiogenesis (b) and fibrous infiltration into pores (c) (x40 magnification).

4.3c Unresorbed scaffold

At 12 weeks, there was less allograft remaining than hydroxyapatite. The amount of allograft reduced from 39% at time zero to between 6 and 21 percent in both the impacted and unimpacted groups (Table 4.3, Fig 4.6). The amount of HA at time zero was 49% and this remained unchanged in the impacted group and reduced minimally in the unimpacted group (Table 4.3, Fig 4.6).

			Remaining Scaffold (%)
Impacted Group	Allograft	without MSCs	17
		with MSCs	21
	HA	without MSCs	49
		with MSCs	49
Unimpacted Group	Allograft	without MSCs	17
		with MSCs	6
	HA	without MSCs	30
		with MSCs	37

Table 4.3: Percentage remaining scaffold in the allograft and HA groups.

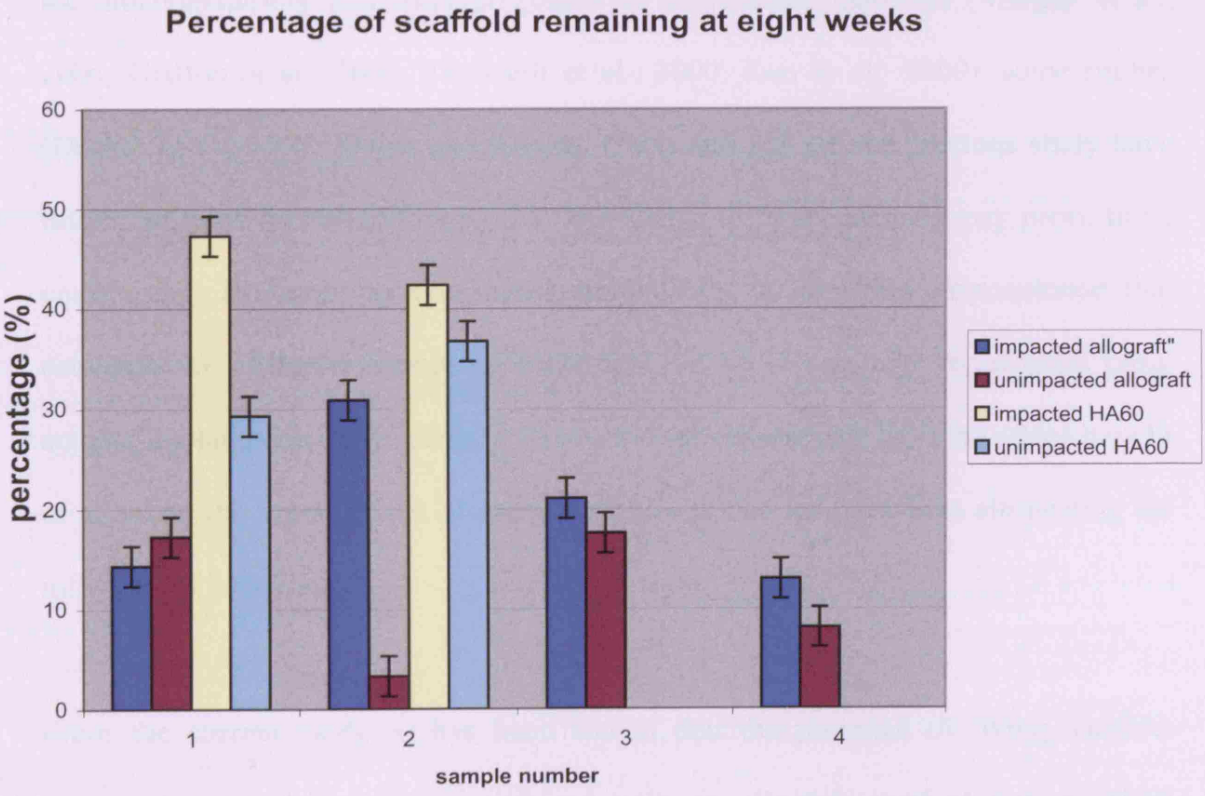


Figure 4.6: Bar Graph demonstrating mean remaining scaffold as percentage field analysed at 8 weeks

4.4 Discussion

There are no animals with the same anatomical, biochemical, physiological and biological characteristics as those of human beings. Non-human primates are considered to be the closest to humans in terms of both their biomechanics and physiology, but on ethical grounds they are rarely used in research in the modern era. For similar reasons, orthopaedic research using canine models and other companion animals have also has declined in recent years. For this reason and given the fact that bone mineral composition and bone healing characteristics in sheep are similar to that of humans (Martini et al., 2001), the ovine model has become increasingly popular in orthopaedic research. Furthermore, sheep are readily available and easy to maintain. Although sheep stem cells have been shown to be a valuable model in determining the biocompatibility and osteointegration of orthopaedic materials (Morgan et al., 2004; Griffon et al., 2001; Torricelli et al., 2000; Kon et al., 2000), some studies (Decker et al., 1979; Deleu and Trueta, 1965) and indeed our previous study have raised concerns that transforming this knowledge to larger animals may prove to be challenging. However, in this initial small study, it has been demonstrated that osteogenicity of tissue-engineered constructs can be successfully investigated using ectopic implantation sites in sheep. Extra-skeletal experiments have the added benefit of allowing the investigation of many variables in one animal whilst eliminating the influence of host bone.

From the current study, it has been shown that the presence of living, culture-expanded mesenchymal cells combined with an allograft scaffold induces more ectopic bone formation than allograft alone. Conversely, a porous calcium phosphate scaffold either alone or combined with mesenchymal cells failed to produce any new

bone ectopically in sheep. The low values of bone formation observed, even in the best samples, are equivalent to amounts seen in other publications (Kruyt et al., 2003). These results have to be interpreted with some caution due to the small sample number.

Our results are in direct contrast to those observed by another study (Kruyt et al., 2003), which showed that a composite graft consisting of a porous HA scaffold combined with osteoprogenitor cells is a prerequisite for in vivo ectopic bone formation in larger animals. However, in that study, the assumption that viable cells were responsible for bone formation contrasts with their finding of reduced bone formation at the scaffold periphery. Cell survival in this area can be expected to be much higher as a result of revascularisation and nutrient diffusion (Pelissier et al., 2003). Furthermore, the investigators of the study were unable to prove that the implanted cells were present in the bone that subsequently formed due to the absence of a reliable tracing method to mark their cells. One observation from the present study which does correlate with that reported by Kruyt et al., (2003) is that the HA scaffold on its own does not induce bone formation ectopically in the larger animal model after a 12 week time period.

An important morphological feature of the scaffold, in in-vivo bone formation by a tissue engineering approach, is the particle size (Mankani et al., 2001). Calcium phosphate particles composed of HA and tricalcium phosphate, with a diameter of 0.1 to 0.25 mm, have demonstrated the greatest bone formation at 4 or 10 weeks when combined with human bone marrow stromal cells and implanted ectopically in nude mice. In contrast, larger (or smaller) particles were associated with less extensive

bone formation. Other authors (Yuan et al., 2002; Fischer et al., 2003) have reported that cells grown on macroporous calcium phosphates devoid of microporosity were unable to produce large amounts of bone in vivo. This has been attributed to the fact that large particles probably create a different macroporosity in the tissue-engineered construct, which could be unfavourable for bone formation. The macroporosity in our samples may have also affected angiogenesis or the speed of blood vessel infiltration in the implanted mass and therefore inhibited survival of the implanted cells and thus osteogenesis occurred within the 2-5mm particles. The presence of bone marrow formation around the microporous particles observed by others (Fischer et al., 2003) may be related to enhanced osteogenesis followed by remodelling and resulting bone marrow formation.

Previous research at our laboratory has shown sheep MSCs are able to survive impaction forces up to 6 kN (Korda et al., 2006). In this and the previous study, a smaller impaction force of 3kN was used to ensure cell survival. One concern that was raised from the last study was that although stem cells survive impaction, their differentiating capacity may be reduced as a result of the impaction process. This theory warrants further investigation. This study has shown that impaction did not have an adverse effect on new bone formation in the allograft group. Moreover, an impacted tissue-engineered allograft construct demonstrated an increase in new bone formation compared with the unimpacted group. It can be reasonable to assume that with the use of autologous plasma as a seeding medium, MSCs survive impaction forces when transplanted in vivo, and continue to differentiate into osteoblasts and stimulate new bone formation.

Conclusion

The osteogenicity of tissue-engineered constructs can be successfully investigated using ectopic implantation sites in sheep. Impacted tissue-engineered allograft constructs demonstrated an increase in new bone formation compared with the unimpacted group. Conversely, an impacted and un-impacted porous calcium phosphate scaffold, either alone or combined with mesenchymal cells, failed to produce any new bone ectopically in sheep. These results have to be interpreted with caution due to the small sample number.

CHAPTER 5

THE EFFECT OF SCAFFOLD ARCHITECTURE ON OSSEOINDUCTION BY MESENCHYMAL STEM CELLS

5.1 Introduction

There is no doubt that a bonding osteogenesis process, in which osteogenic precursor cells become attached to the biomaterial implant surface, aggregate, proliferate, differentiate, produce bone matrix and ossify, has been observed in calcium phosphate biomaterials (Dekker et al., 1998). However, in the study by Kruyt et al (2003) significantly less bone was formed when cells were combined with HA60/400 (HA with 60% interconnected macroporosity and an average pore size of 400μm), as used in the pilot study in Chapter 4, than with HA70/800 (HA with 70% interconnected macroporosity and an average pore size of 800μm). The authors could not provide a satisfactory explanation to account for this difference. In a mice ectopic model, different apatite-containing scaffolds of similar morphology and porosity demonstrated marked differences in their ability to support osteoinduction by implanted human MSCs (Harris and Cooper, 2004). It may be that the induction of human MSCs along the osteoblastic lineage may be dependent, in part, on the local microenvironment established by the scaffold chemistry and interactions with the host (Harris and Cooper, 2004). From the above studies it appears that the architecture of the scaffold influences the proliferation and differentiation of stem cells and could account for our observed differences in new bone formation. Although some investigators (Mendes et al., 2003) have demonstrated extensive osteogenesis using stem cells loaded onto porous HA granules, the majority of studies have assessed TE constructs with MSCs seeded onto blocks of porous ceramic bone substitutes (Bruder et al., 1998; Kon et al., 2000; Kruyt et al., 2003). In fact, some authors advocate that bone grows in porous hydroxyapatite only if constructed in block forms and fails to grow in identical porous hydroxyapatite in granular form (Ripamonti, 1996; Ripamonti et al., 1993; Ripamonti, 1991). In addition, experiments in rodents have

shown that even when granular hydroxyapatite has been pre-treated with BMPs, bone does not form (Ripamonti, 1992). Similar results have also been observed in biopsies obtained after maxillo-facial augmentation in humans (Hjorting-Hansen et al., 1990). One reason for the observed lack of osteogenesis could be due to micromotion between the individual granules and the adjacent bone interface. Micromotion has been shown to inhibit bone growth into porous biomaterials leading to fibrous union (Cameron et al., 1973).

In the pilot study described in Chapter 4, in both the impacted and unimpacted groups there was more new bone formation when allograft was seeded with mesenchymal stem cells compared with allograft alone. However, the low values of bone formation observed in the study is equivalent to amounts observed by other investigators (Kon et al., 2000; Kruyt et al., 2003). A possible reason for this could be that although stem cells survive the impaction, their differentiating capacity may be reduced as a result of the impaction process. One approach to address this theory would be to investigate the bone-forming capacity of osteoblastic cells and compare it with undifferentiated mesenchymal stem cells. Techniques for directing the commitment of MSCs into bone cell lineage to produce osteoblastic like cells (OBLC) which differentiate into osteoblasts, thus resulting in new bone formation, have been successfully developed (Jaiswal et al., 1997; Haynesworth et al., 1992) and reproduced in our laboratories (Kalia et al., 2006).

The differentiation of stem cells can be divided into three stages: (i) a proliferative phase, (ii) the period of matrix deposition and (iii) the mineralisation phase (Duplomb et al., 2007). Zur Nieden et al (2003) studied the expression pattern of osteoblastic

markers and showed that there is an increase in alkaline phosphatase (ALP) activity during the matrix deposition stage and a maximal expression of osteocalcin, an osteoblast-associated matrix gene, at the start of the mineralisation phase. In addition, the capacity of osteoblasts to mineralise *in vitro* can be visualised by von Kossa or Alizarin red staining (Jaiswal et al., 1997; Duplomb et al., 2007).

The study in Chapter 2 has demonstrated that the amalgamation of HA with allograft in impaction grafting presents a number of potential advantages. To our knowledge, there have been no previous studies to assess the suitability of a mixed allograft / HA scaffold for the delivery of mesenchymal stem cells. The previous study has demonstrated that the osteogenicity of tissue-engineered constructs can be successfully investigated using an ovine extra-skeletal model.

5.1a Hypothesis

This study will explore the hypothesis that in an ectopic sheep model (a) impacted allograft seeded with MSCs or osteoblast like cells (OBLCs) will produce more new bone than impacted allograft only; (b) impacted HA / allograft mix (50:50) seeded with MSCs or OBLCs will produce more new bone than impacted HA / allograft only and (c) MSCs seeded onto an HA block will produce more new bone than an HA block alone.

5.1b Objectives

Ectopic bone formation will be assessed by histology and histomorphometry of non-decalcified sections.

5.2 Methods

5.2a Differentiation of stem cells for osteogenesis

The purification, culture and expansion of MSCs have been described in the previous chapters. During passage 2, MSCs were cultured in an osteogenic medium (DMEM supplemented with 10mM β-glycerophosphate, 0.05mM ascorbic acid, and 100nM dexamethasone) for a total of 14-21 days to produce osteoblastic like cells (OBLC). This differentiation was confirmed by an increase in ALP activity of the cells, expression of osteocalcin and positive staining with the von Kossa method.

5.2b Cell Proliferation Assay

To determine seeding efficiency and cell increase after culturing in the osteogenic medium, the DNA content the cell layer was measured using the fluorescent dye Hoescht 33342 (Sigma, B2261, U.K.). This method is based on the observation that when the Hoescht dye binds to DNA it becomes fluorescent. The amount of fluorescence is proportional to the amount of dye bound and thus, to the amount of DNA (number of cells) present in the sample. In brief, each well received 100μl of 5 ng/ml Hoechst 33342 in phosphate-buffered saline (PBS) and the plate was incubated at 37 °C for 20 min. Fluorescence was measured using a fluorescence reader for microplates (Ascent, Labsystems, UK) at 350excitation and 450nm emission wavelength. Absorbance values were converted into absolute cell numbers based on established standard curves.

5.2c Alkaline Phosphatase Assay

Alkaline Phosphatase (ALP) enzyme activity of the cell layer was measured in triplicate cultures by rinsing twice with Tyrode's balanced salt solution, and then

incubating the cells with 5mM p-nitrophenyl phosphate (Randox Laboratories Ltd, U.K.) in 50mM glycine, 1mM MgCl2, pH 10.5, at 37°C for 5 to 20 minutes. ALP activity was calculated after measuring the absorbance of p-nitrophenol product formed at 405 nm on a microplate reader (Cobas Bio, Roche, UK).

5.2d Von Kossa Staining

Cells cultured in osteogenic medium were stained for mineral by the von Kossa method (Jaiswal et al., 1997). Briefly, fetal calf serum was serially diluted out of the medium by exchanging 80% of the medium several times with serum-free medium. The cells were fixed in 5% formaldehyde and then washed several times with PBS to remove any remaining serum. The fixed cells were incubated in 100% ethanol at 4°C for about 10mins. The ethanol was then removed and the fixed cells were incubated in 0.5ml 5% silver nitrate for 10mins under UV light at 254nm. The cells were then rinsed 2-3 times in distilled water, incubated in 5% sodium thiosulphate for 5mins and then rinsed with water. Only cells receiving osteoblast medium stained positive and turned dark.

5.2e Expression of Osteocalcin

Osteocalcin expression was measured by ELISA (Intact Human Osteocalcin K EIA kit, catalog number BT-460, Biomedical Technologies, Inc., Stoughton, Me.). Media obtained from osteoblasts was examined for the presence of secreted osteocalcin. Briefly, conditioned medium (2 ml of medium from 40,000 cells after 72 hours) was collected and 20 µl of each medium was used for the assay.

5.2f Experimental design

Six skeletally mature ewes (mean body weight 70.9 ± 3.5 kg) were used, for which approval was given by the local ethics committee. The study was divided into eight treatment groups (Table 5.1). Allograft was prepared and stored as described in Chapter 2. ApaPore 60, 2-5mm diameter, was used as HA granules whilst the 10mm granules were used as blocks. The allograft and HA samples were sterilised by gamma irradiation as described previously. MSC's and OBLCs were seeded at 10×10^6 cells/g onto the scaffold. One unit of each treatment group was randomly allocated to one of four implantation positions in the paraspinal muscles of the sheep from which the MSCs were derived (autologus implantation). The surgical procedure was similar to that described in the initial study. All the animals were euthanased 12 weeks after surgery with an overdose of intravenous Pentobarbitone.

5.2g Histology and histomorphometry

After explantation with the associated surrounding muscle, the implants were investigated for ectopic bone formation by histology and histomorphometry of non-decalcified sections. The samples were prepared for histology as described in the previous chapters. The sections were examined using a fluorescence/light microscope with a double filter block. Digital photographs through the entire area of the sample were taken using a low power x40 magnification. Area of new bone formation and remaining scaffold were quantified using the Image Tool 3.0 software (UTHSCSA, Texas, USA). The percentage of new bone formation was expressed as the amount of new bone over total area available for new bone generation. New bone in contact with the scaffold (allograft or HA) was determined as a percentage by defining

whether bone or fibrous tissue was in contact with the edge of the scaffold where it intersected with lines on an overlay grid pattern of 500μm.

	Sample composition	Sample type
Impacted group	allograft alone	Control
	allograft with MSCs	Test
	allograft with OBLCs	Test
	allograft / HA (50:50) mix	Control
	allograft / HA (50:50) mix with MSCs	Test
	allograft / HA (50:50) mix with OBLCs	Test
Unimpacted group	HA block	Control
	HA block with MSCs	Test

Table 5.1: Composition of the eight treatment groups used in this study

5.2h Statistical Analysis

Statistical analysis was carried out using the SPSS (v10.1) statistical software. A multivariate study of variance was used to analyse the difference between the groups. If a significant difference was observed ($p < 0.05$), post hoc analysis was used to identify the differences between individual groups.

5.3 Results

5.3a Allograft group

There was no statistical difference in new bone formation amongst the three different groups, but there was a trend towards an observed mean increase in new bone formed when allograft seeded with MSCs was compared with allograft alone or allograft seeded with OBLCs (Fig 5.1).

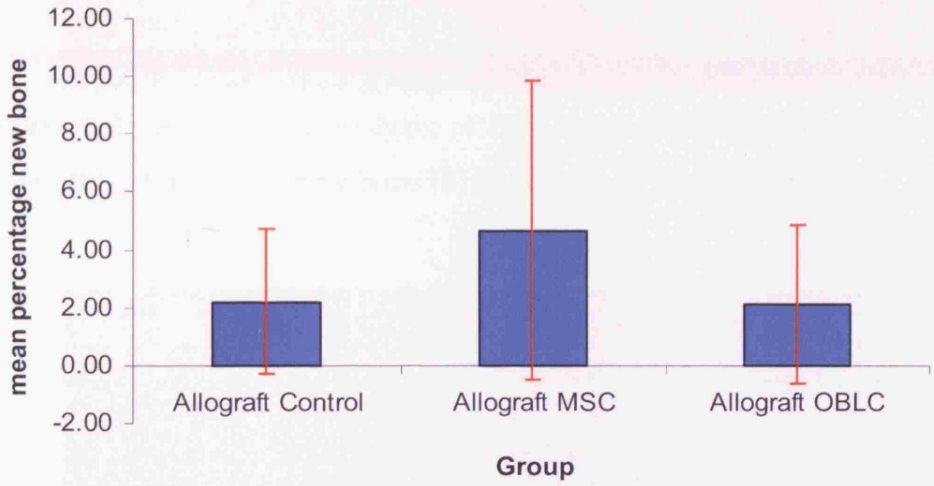


Figure 5.1: New bone formation in the allograft group (mean ± S.D.).

New bone formed bridges between individual pieces of allograft, with osteocytes and lacunae being clearly visible (Figs 5.2 & 5.3). New bone was differentiated from allograft as it stained vividly red, whereas allograft stained less intensely. Surfaces of new bone were covered in a layer of osteoblasts and surrounded by unmineralised osteoid. Osteoclasts demonstrated active areas of resorption (Figure 5.3). At time zero, 39% of the defect was filled with allograft, but at twelve weeks the majority of scaffold had been resorbed in all three groups.

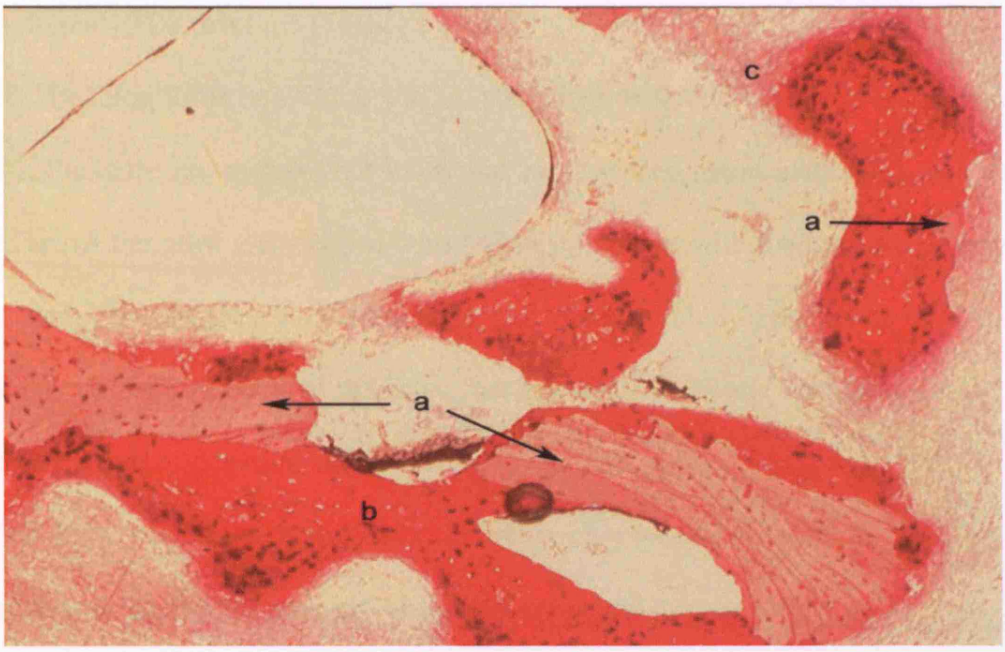


Figure 5.2: Histological analysis of MSC seeded allograft (x40 power). Areas of allograft (a) bridged by new bone (b) with areas of ossification (c)

Figure 5.3: Histological analysis of MSC seeded allograft (x100 power). Allograft (a) with new bone forming within pores (b) with osteocytes (c) and osteoblasts (d) being clearly visible

5.3b Allograft:HA mixture (50:50)

As with the allograft group, there was no statistical difference in new bone formation amongst the different groups, but there was an observed mean increase in new bone formed when the graft was seeded with MSCs compared with the graft alone or graft seeded with OBLCs (Fig 5.4). New bone was demonstrated in the pores of HA (Fig 5.5) and occasionally bridged granules, but minimal new bone was seen around the surfaces of the HA granules. At time zero, 48% of the defect was filled with scaffold, of which 41% was allograft and 59% HA. At twelve weeks, most of the allograft had been resorbed, whilst the majority of the HA remained unresorbed.

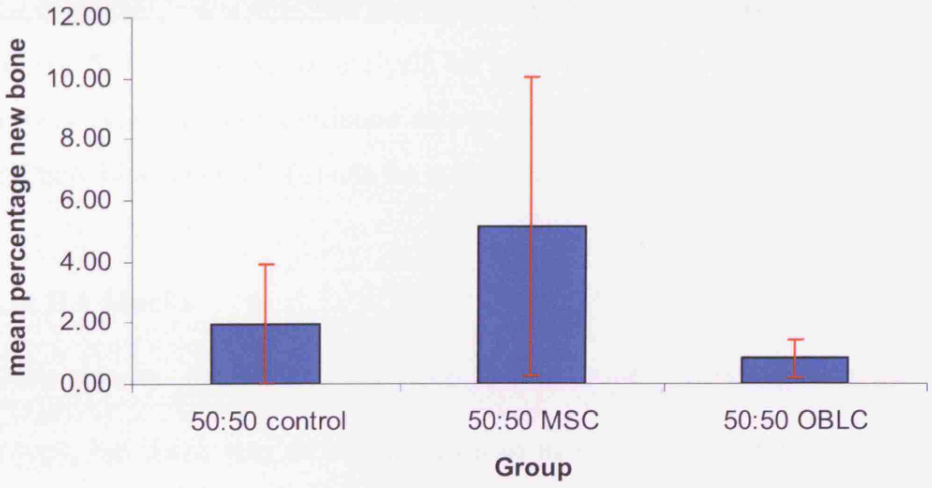


Figure 5.4: New bone formation in the allograft:HA (50:50) group (mean ± SD).

Figure 5.5: Histological analysis of MSC seeded on Allograft: HA mixture (x100 power). HA (a) with evidence of new bone formation within pores (b). Osteocytes and new blood vessels (c) can be seen within the new bone.

5.3c HA blocks

There was no statistical difference in new bone formation amongst the HA block groups, but there was an observed mean increase in new bone formed when the HA block was seeded with MSCs compared with the HA block alone (Fig 5.6). At time zero, 52% of the defects were filled with allograft and this remained unchanged at 12 weeks.

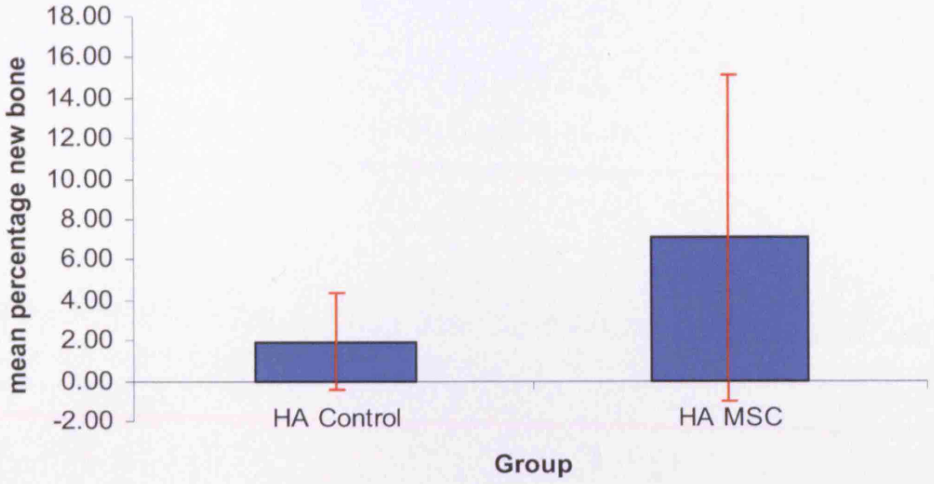


Figure 5.6: New bone formation in HA blocks (mean ± S.D.)

5.4 Discussion

This study confirms and validates the results of the preceding two experiments that stem cells survive the impaction process without any apparent adverse effect on their differentiating capacity. It should be noted that any results from this study should be interpreted with caution due to the small sample size. As in the study described in Chapter 4, cells combined with an allograft scaffold induced more ectopic bone formation than allograft alone. Although this increase in bone formation was not statistically significant, retrospective power analysis revealed it would require twelve animals within the study to demonstrate a difference with a 95% confidence interval. In the earlier study, a porous calcium phosphate scaffold combined with MSCs failed to produce any bone ectopically, but this experiment has shown that cells loaded onto a 50:50 mixture of allograft and HA induces the same amount of new bone formation, as with cells seeded on an allograft scaffold alone. The use of an amalgamated scaffold has the benefit of combining the osteoinductivity of allograft with the structural stability of hydroxyapatite. It may be possible to use greater amounts of HA in a mixed scaffold without altering the functional capacity of stem cells, thus reducing the amount of allograft used and alleviating the problems associated with its use. More new bone formation was noted in the HA block group seeded with MSCs compared with the other groups. This could be because new bone was measured over the entire area of the graft and this probably naturally skews the results in favor of the block group as there was more scaffold per unit volume.

The addition of Osteogenic Supplements (100nM β-glycerophosphate, 0.05mM ascorbic acid and 10nM dexamethasone) to MSCs is a reliable and well established technique for inducing rapid osteogenesis (Jaiswal et al., 1997; Haynesworth et al.,

1992). Furthermore, the expression of alkaline phosphatase provides a reliable tracing method to mark mesenchymal stem cells. It is therefore plausible to assume that MSCs cultured in such a medium would differentiate down the osteoblastic lineage. However, it could be speculated that MSCs cultured in an osteogenic medium may become more fragile and are unable to withstand the forces of impaction. This would account for the observed lack of new bone formation, in this study, with the use of a TE construct with OBLCs.

The majority of studies investigating osteogenesis using stem cells have assessed TE constructs with cells loaded onto blocks of HA (Bruder et al., 1998; Kon et al., 2000; Kruyt et al., 2003) because of concerns that bone fails to grow in porous hydroxyapatite in granular form (Ripamonti, 1996; Ripamonti et al., 1993; Ripamonti, 1991). The initial study in Chapter 4 failed to show new bone formation using cells loaded onto porous granular HA, but the current study has demonstrated observable osteogenesis when stem cells are seeded onto blocks of HA. This has a major clinical implication as most of the commercially available HA is produced in granular form. Furthermore, the impaction technique can not be used with blocks of hydroxyapatite.

5.5 Conclusion

The addition of MSCs to allograft or a 50:50 mixture of Allograft:HA by impaction grafting enhances the amount of new bone formation when compared with unimpacted constructs. There is an observed lack of new bone formation with the use of a TE construct with differentiated osteoblastic like cells. The use of a calcium phosphate scaffold, either alone or combined with mesenchymal cells, induces bone growth only when it is constructed in block form and not in identical porous hydroxyapatite in granular form. This has major clinical implications as most of the commercially available HA is produced in granular form and impaction grafting can not be used with blocks of hydroxyapatite.

DISCUSSION

Revision total hip arthroplasty in the presence of extensive femoral bone loss poses a considerable challenge to the orthopaedic surgeon and probably accounts for the inferior results observed compared with the primary procedure. For this reason bone grafts and bone graft substitutes are increasingly being used to replenish the bone loss that occurs with loosening. One method that has been successfully used to treat cavitary defects, in the proximal femur prior to the insertion of a revision femoral prosthesis, has been the use of impaction allografting, whereby morsellised cancellous allograft is impacted into defects to provide immediate mechanical function. Whilst in structural grafts, bone ingrowth does not usually exceed 2 to 3mm, in impacted morsellised allografts there is greater bone integration.

Impaction allografting is the only technique, to date, that has been shown to reverse the loss of bone stock caused by osteolysis. However, when evaluating long-term outcome studies on impaction grafting there is a wide variation in results, some as high as 100% survival at 10 years (Schreurs et al., 2005), others as low as 28% survival at 15 years (Jeffery et al., 2003). There are many factors responsible for this variation. Firstly, the degree of osteolysis varies considerably amongst published reports. In some series, only cases with severe advanced bone loss have been included (Masterson et al., 1997; Meding et al., 1997; Leopold et al., 1999), whilst in other studies cases with such severe bone deficiencies have specifically been excluded (Gie et al., 1993; Ling, 1997). In some studies, the degree of bone loss has not been defined at all (Eldridge et al., 1997). Secondly, it is well understood that the success of impaction grafting depends upon achieving adequate initial stability followed by a biological response of graft incorporation and remodelling. Surgeons have tried to manipulate the graft characteristics to improve one or both of these factors. The ideal graft has not yet been defined. Fresh-frozen femoral heads are the most frequently

used, but concern remains over the potential for transmission of disease and for this reason the use of processed bone (freeze dried or irradiated bone) is attracting some attention. However, clinical studies using processed bone have shown inconsistent results and currently a review to compare the clinical effectiveness of processed bone against fresh frozen bone is being conducted. The ideal size of bone chips for the acetabulum has been well defined but evidence on the femoral side is less certain. Washing the graft has been shown to be beneficial but the most efficient method has not been established. Further work is needed to define the most appropriate production and processing techniques of the graft in order to generate a mechanically stable construct conducive to biological incorporation and remodelling. Thirdly, there are a vast number of confounding variables in the published reports that again limit valid comparisons. For example, clinical series have differed with respect to stem geometry, cement type, surgical approach and post-operative care of patients (Leopold and Rosenberg, 2000; de Roeck and Drabu, 2001). Finally, methods of patient analysis, including clinical and radiographic assessment and follow-up duration, have differed markedly among published reports (Leopold and Rosenberg, 2000).

Despite these variations, numerous studies have shown excellent clinical, radiological and histological results, demonstrating that impaction grafting with allograft can reverse loss of bone stock, with bone graft being replaced by new bone in both humans and in animal models (Toms et al., 2004). The purpose of this research was to investigate whether the addition of bone graft substitutes with allograft induces similar patterns of bone formation as allograft alone. The concept of adding biomaterial to bone allograft is attractive because it reduces dependency on donor supply, minimises the complications associated with the use of bone grafts and raises

the possibility of improving the biomechanics of the graft. Furthermore, the use of an amalgamated graft has the benefit of combining the osteoinductivity of allograft with the structural stability of biomaterials. Besides an improvement of biomechanical properties, expansion or replacement of allograft with synthetic agents would have biological and economical advantages. Indeed, one of the current limitations of impaction grafting is that it relies on the use of allogenic bone. Substituting bone with synthetic granules would address the concerns over transmission of pathogens and shortage of supply.

ApaPore 60 is a porous hydroxyapatite that has been chemically and structurally modified to optimise host bone ingrowth in impaction whilst maintaining structural integrity. It has high levels of interconnectivity between micro and macropores. This network allows rapid bone ingrowth, promotes revascularisation, which is essential to healthy bone, enhances long-term graft stability and provides a continuous host bone/graft composite. Besides an improvement of biomechanical properties (Buckland T and Lawes P., 2002), expansion of allograft with ApaPore 60 would have biological and economical advantages. Furthermore, augmenting bone graft with ApaPore 60 would reduce the risk of transmission of pathogens and address the shortage with the supply of allograft (Galea et al., 1998). This thesis investigated if femoral impaction grafting using bone graft substitutes would yield similar results to that observed with allogenic bone.

In Chapter 2, I examined whether the use of ApaPore in various combinations with allograft would be biologically effective and functionally stable using a cemented impaction grafting technique in an ovine hemiarthroplasty model. The different

treatment groups were compared by measuring the ground reaction forces (GRF) passing through both the operated and non-operated hind limbs pre-operatively and at 8, 16 and 24 weeks post surgery. GRF results are a measure of implant function and how well the animal uses its operated limb and a persistent reduction in function can be evidence of implant loosening. Furthermore, a failure to regain function after surgery implies that the loads passing through the implant and the associated micromotion may not be representative of a normal healing process, which gives anomalous results in terms of bone ingrowth. In addition to GRF, bone formation rates within each of the experimental groups were also measured. This allowed quantification of the rate of new bone formation within each of the groups, thus permitting comparison of the osteogenic potential of the different groups.

Results from this study demonstrated a successful fixation of the femoral stem six months post surgery using impaction grafting, irrespective of the graft combinations. All hips maintained functional stability when higher amounts of HA mixture was used, with no significant differences identified between experimental groups when limb function was assessed and compared. The osseoconductive properties of ApaPore were confirmed by histological and backscattered SEM analysis, which demonstrated a remodelled endosteal surface with abundant bone growth in direct contact, within and around ApaPore granules, forming a network of mature lamellar bone adjacent to the implant and within the original medullary cavity. Significantly increased bone area was measured in Groups 1 (50:50) and 3 (90:10) compared with Groups 2 (70:30) and 4 (80:10:10), but there was no significant difference between groups 1 and 3.

A study by Buckland & Lawes (2004) comparing the use of allograft alone with 50:50:allograft in impaction grafting demonstrated that the 50:50 group had 50% greater mechanical stability, 50% decrease in subsidence and a significant reduction in the variability of the mechanical properties of the graft material. My study has shown that successful fixation can be achieved with up to 90% ApaPore. It is doubtful whether the allograft in the 90:10 graft mixture contributed to the stability of the graft, but this theory remains unproven. Other studies have shown that due to the poor structural integrity of hydroxyapatite and inferior mechanical properties in comparison to bone graft, impaction grafting with pure HA is not possible and amalgamation with allograft is necessary (Verdonschot et al., 2001; Silva et al., 2005). Based on these assumptions, my study did not examine the osteogenic potential of 100% ApaPore, but given the encouraging results observed with a 90:10 HA:allograft mixture, impaction grafting using pure HA granules can be investigated. This has important clinical implications as hydroxyapatite is readily available, easy to use in surgery and is not associated with the adverse effects encountered with allografts.

Actifuse is a phase-pure 80% porous calcium phosphate material in which the phosphate groups have been selectively replaced with silicate ions. Studies have shown that silicon is a significant dietary trace element for healthy bone formation (Carlisle, 1981; Schwarz and Milne, 1972) and stimulates osteoblastic differentiation to form new bone (Patel et al., 2002). Moreover, the surface chemistry of Actifuse has been shown to enhance adsorption and activation of the proteins involved in osteogenesis, leading to much more rapid and better quality bone formation than with hydroxyapatite alone (Hing et al., 2006). In my study, however, the impaction process

was shown to alter the mechanical and biological properties of Actifuse differently to that of phase pure hydroxyapatite. It may be that the Actifuse particles fracture under impaction or deform elastically and are damaged to a significant level. In-vitro studies evaluating the time dependent mechanical properties of Actifuse would provide answers to this question. Furthermore, Actifuse is currently only supplied with 80% porosity, however bone substitutes with large porosity have been shown to deform more easily during impaction grafting than less porous biomaterials (Verdonschot et al., 2001). Although the exact porosity of biomaterials that mimic the biomechanical properties of bone graft is not known, studies have shown that a high porosity is a desirable feature of synthetic bone substitutes as it enhances neovascularisation, which is essential for osseointegration (Virolainen et al., 1997). For this reason, most biomaterials are produced with at least 60% porosity. Future studies could investigate the effect of the impaction process on a graft formed from a mixture of ApaPore and Actifuse with 60% porosity and even pure Actifuse with 60% porosity. The results would provide a greater insight into how these variables affect the biological and mechanical response of silicate-substituted hydroxyapatite.

There were limitations to the study described in Chapter 2, and any conclusions drawn from it need to take these into account. Firstly, although the primary ovine hip replacement model has been successfully used to investigate a revision scenario (Blom et al., 2005; McGee et al., 2004) there may be aspects of the tissue environment prior to revision hip surgery, in humans, that are not represented in this model i.e. endosteal erosions, residual inflammatory tissue and compromised host bone quality (Verdonschot et al., 2001). Secondly, there may have been discrepancies in the impaction grade between the different groups due to the effects of variable

particle size distribution and the reduced compressibility of hydroxyapatite compared to allograft on the level of impaction (Verdonschot et al., 2001). However, to minimise the disparity in impaction grade, all specimens were prepared and manually impacted by the same person.

As advances in molecular biology are made, there has been a gradual move from a tissue approach to a more cellular approach in providing a more efficient means of reconstituting bone stock. Bone marrow contains a population of cells capable of differentiating into bone, cartilage, muscle, tendon, and other connective tissues. These mesenchymal stem cells (MSCs) have successfully been differentiated into osteoblasts, at our Institute. Furthermore, studies have shown that MSCs are able to survive the standard impaction forces encountered at revision hip surgery and their differentiating capacity does not appear to be reduced as a result of the impaction process (Korda et al., 2006).

The work in chapter 3 of this thesis exploited the use of impaction grafting using a composite graft formed from the osteoconductive matrix of allograft and/or granular HA combined with mesenchymal stem cells (MSCs), which provide osteoinductive and osteogenic properties. In this way, the allograft and/or HA acts as a delivery system and scaffold for the MSCs, allowing the passage of osteoblast progenitor cells to the graft site and facilitating bony ingrowth. Results showed that the addition of MSCs to allograft alone or a 50:50 mixture of allograft/granular HA by impaction grafting enhanced the amount of new bone formation when compared with unimpacted constructs. An ovine metaphyseal femoral unicortical bone defect model was selected for this study and although this is a suitable model which simulates some of the environmental characteristics encountered in revision hip arthroplasty, it

does not mimic the loading conditions encountered clinically. Further work evaluating a composite graft composed of allograft/HA seeded with MSCs in an ovine hemiarthroplasty model would provide valuable information as hip forces in sheep correlate well with those in humans (Bergmann et al., 1984). Thus, functional loading of the graft-composite would be similar to that encountered in the clinical setting and would indicate whether this technology is transferable to humans.

In Chapter 3, a metaphyseal femoral condyle defect model was used to investigate the osseoinductive capacity of impacted TE constructs. Although this model has several advantages, the environment does not simulate the conditions encountered clinically during a revision situation where impaction grafting would be used. For example, this area has a good blood supply and does not mimic the poor bone stock encountered in a clinical revision situation. Furthermore, within the sheep condyle the environment is very conducive to bone formation and any effect that the stem cells would have had may have been masked. To rule out osteoconduction or periosteal bone formation (Bruder et al., 1998; Kon et al., 2000) as disturbing and overriding mechanisms of bone formation, ectopic implantation sites have been used successfully to investigate the bone-forming osseoinductive capacity of tissue-engineered constructs implanted in goats (Kruyt et al., 2003). In Chapter 4, I explored the hypothesis that the addition of MSCs to allograft or HA granules would enhance the amount of new bone formation when compared with allograft or HA granules alone in an ectopic sheep model. An extra-skeletal model would indicate the osseoinductive nature of the combined scaffold and stem cells and has the added benefit of allowing the investigation of many variables in one animal.

Results showed that in both the impacted and un-impacted groups, there was more new bone formation when allograft was seeded with mesenchymal stem cells compared with allograft alone. Conversely, an impacted and un-impacted porous ApaPore scaffold, either alone or combined with mesenchymal cells, failed to produce any new bone ectopically in sheep. The low values of bone formation observed, even in the best samples, were equivalent to amounts seen in another publication (Kruyt et al., 2003). These results have to be interpreted with some caution due to the small sample number.

My results are in direct contrast to those observed by Kon et al (2000) who showed that cells loaded onto porous HA scaffold conducted a far more extensive bone formation than cell free implants in tibial defects in sheep. However, there are several noticeable differences between their study and this one. Firstly, they used un-impacted cells. Secondly, they used an external fixator to achieve mechanical stability and this could potentially cause mechanical stimulation of both implants and bone resected ends. Thirdly, they used fibrin as a cell sealant, which itself has been shown to promote osteogenesis from the host osteoprogenitor cells (Fortunato et al., 1997). Finally, the animals in their study were euthanased at 8 weeks whereas in my study they were sacrificed at 6 weeks. It could be speculated that more bone formation could have been observed in my study if it was conducted over a longer period.

Another fundamental difference between the study by Kon et al (2000) and that described in Chapter 4, is that they investigated a scaffold consisting of HA cylinders and not granules, as used in my study. It therefore appears that the morphology of the scaffold is crucial in tissue engineering and this formed the basis of Chapter 5. In this study, I investigated the potential of the use of a composite graft formed from blocks

of HA seeded with MSCs and demonstrated observable osteogenesis. This observation reflects the results of the majority of studies that have assessed TE constructs with MSCs seeded onto blocks of porous ceramic bone substitutes (Bruder et al., 1998; Kon et al., 2000; Kruyt et al., 2003). In fact, Ripamonti et al. (1991, 1993, 1996) found bone induction only in block-shaped HA with cavities and failed to grow in identical porous hydroxyapatite in granular form. They referred to this morphologic prerequisite as geometric induction. It is possible that in block-shaped HA with cavities there occurs an accumulation of factors that are needed for induction and/or differentiation of cells (Yamasaki and Sakai, 1992). Furthermore, such a micro-environment may favour calcium phosphate dissolution and re-precipitation, a mechanism postulated by some investigators (Kruyt et al., 2003; Yuan et al., 2002) to be responsible for bone induction. This induction theory may even be supported by the finding that osteogenesis inside arteriosclerotic plaques only occurred as a final stage, inside calcified (hydroxyapatite) plaques (Deneke et al., 2001). In the context of this thesis, this has major clinical implications as most of the commercially available HA is produced in granular form and impaction grafting can not be performed with blocks of hydroxyapatite.

Recently, the use of bone morphogenic proteins (BMPs), in the treatment of osteolysis has attracted much attention. BMPs are a family of osteoinductive growth factors that can initiate endochondral bone formation, presumably by stimulating osteoblast progenitor cells, and by enhancing bone collagen synthesis. Recombinant human bone morphogenic protein-7 (BMP-7 or OP1) has been shown to promote the incorporation of morsellised allograft bone into new host bone in an animal model (Salkeld et al., 2001; Jensen et al., 2002) and has shown good results in treatment of fracture non-unions and spinal fusion surgery in humans (Friedlaender, 2001; Sandhu

and Boden, 1998). Initial reports of the use of OP-1 in association with morsellised cancellous allograft to treat major bone loss during revision hip surgery have shown promising results (Cook et al., 2001). However, in a loaded bone grafting situation, OP-1 was not shown to promote the incorporation of the graft bone into host bone in large animal studies (McGee et al., 2004; Buma et al., 2007; Jeppsson et al., 2003; Tagil et al., 2003). In humans, the addition of OP-1 to morsellised allograft during impaction grafting has not been shown to improve the early fixation of either the acetabulum or femoral component at a five year follow-up (Karrholm et al., 2006). The most likely explanation for these disappointing results is that although BMPs enhance new bone formation, they also accelerate bone resorption in the early phase after grafting (Jensen et al., 2002; Kaneko et al., 2000). It could be speculated that an impaired bone bed, as encountered during revision hip arthroplasty, changes the balance between bone formation and resorption towards the latter. Clearly, uncontrolled graft resorption in human femoral impaction grafting could lead to implant subsidence and loosening and therefore the use of BMPs in impaction bone grafting can not be advocated at present.

These results confirm that the key to success with impaction grafting is to obtain sufficient primary stability of the tissue engineering construct. In this thesis, the addition of MSCs to an allograft or a 50:50 allograft:HA scaffold by impaction grafting enhanced the amount of new bone formation when compared with unimpacted constructs, whereas an impacted ApaPore scaffold combined with MSCs failed to produce any new bone. It therefore appears that allograft provides stability for the MSCs to allow bone formation, but HA granules do not. One reason for the observed lack of osteogenesis with HA granules could be due to micromotion between the individual granules and the adjacent bone interface. Micromotion has

been shown to inhibit bone growth into porous biomaterials, leading to fibrous union (Cameron et al., 1973; Lindholm et al., 1994). For example, Snyders et al. (1993) evaluated the use of negatively charged dextran beads with and without the use of calcium sulphate slurry as a binding agent in 5mm diameter calvarial defects in rat. The combination of the two materials promoted better bony repair, whilst the dextran beads used alone produced mostly fibrous tissue with less bone in the wound than even the controls. The authors attributed the success of the combined treatment to the binding effect of the calcium sulphate and its ability, after setting both, to maintain the material at the site without movement and to retain the space, preventing soft tissue collapse into the wound area. Lindholm et al (1994) have also provided evidence of poor osteoconductivity of granular material in the calvarial model and demonstrated that motion of HA granules initiated the formation of inflammatory granulation tissue, which impeded new bone formation. These findings seem to suggest the need for sufficient stability of implanted biomaterials during healing in order to allow for bone formation.

In conclusion, this thesis has demonstrated that HA is a suitable bone substitute to augment allograft and may be used to replace bone graft in impaction grafting of a femoral component. This has important clinical implications as it is readily available, easy to use in surgery and not associated with the adverse effects encountered with allografts. The use of MSCs in the treatment of osteolysis holds great potential, but further work is required to assess if this technology is transferable to humans. If successful, it could have a substantial public health impact and would improve the functional results of thousands of patients undergoing revision joint arthroplasty.

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